SYSTEMATICS AND THE PHENOTYPIC EVOLUTION OF PAPUAN MICROHYLID FROGS

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ABSTRACT

Understanding how biodiversity is generated is a central task of evolutionary biology. Many studies have established that natural selection or stochastic processes, like population fragmentation via biogeographic barriers, play a major role in generating biodiversity. However, much less attention has been paid to how these processes interact. A promising group in which to study the generation of biodiversity is the *Asterophyinae* frogs of Paupa New Guinea. Recently, it has been discovered that this is a hyper-diverse group of frogs, both in species number and ecological habit. Field workers have proposed five different ecological types based on where they are found: tree, shrub, ground, semi-aquatic, and subterranean. However, no formal evolutionary or ecological studies have yet been conducted. Here, I explore adaptive and stochastic processes in the Asterophryine frogs by first constructing a time-calibrated phylogeny of the clade to determine the intergeneric relationships and ecological evolution of the group. I then investigate the morphological and performance evolution of 28 species with over 500 individuals to determine if these phenotypes are evolving in response to natural selection to microhabitat. I find that ecological novelty arose early in the group's history and is tied to the rise of the Central Mountains of New Guinea. It also appears that amalgamation of offshore land masses onto New Guinea lead to bursts of species divergence but less so to ecological transitions. Furthermore, I find that the species have evolved specialized morphologies that match microhabitat-use, providing support for the reality of "ecomorphs". These morphologies are also convergent so that species of the same ecomorph evolved similar morphologies, independent of phylogenetic relationships. Last, I find that performance capabilities in terms of jumping, climbing and swimming

differ between ecomorphs and these differences are imparted by specialized morphologies. This correspondence between ecology, morphology, and performance capabilities independent of phylogeny, provides strong evidence that selection is an important force in the phenotypic diversification of the lineage. Overall, I demonstrate that the phenotypic diversity seen in the *Asterophryinae* is driven by selection to microhabitat. Therefore, both adaptive and biogeographic processes were needed to generate the great diversity seen in the *Asterophryinae* today.

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CHAPTER 1. INTRODUCTION

Evolutionary biologists have always been fascinated by the processes that generate and maintain biodiversity. Many champion the view that selection is the overwhelming force that is producing biodiversity (e.g., Simpson, 1944; 1953; Endler, 1986; Williams, 1996; Schluter, 2000). For example, Darwin's finches possess a myriad of beak shapes that differ in length, width, depth, and curvature. These shapes are associated with a particular food type and provide an advantage in processing the food according to the hardness and size of food consumed (Schluter and Grant, 1984). This correlation between morphology and ecology in providing a performance advantage is referred to as ecomorphology (Arnold, 1983). Similarly, the *Anolis* group of the Caribbean utilizes a variety of microhabitats that range from tree canopies, to tree trunks, to grass and bush specialists (Williams, 1972). Selection has driven morphology to match perch type so that locomotor capabilities are tightly associated with microhabitat use (Losos, 1990). These classic examples of the ecology, morphology, performance paradigm provide compelling evidence for the power of adaptation in producing phenotypic diversity (Arnold, 1983).

However, others argue that stochastic processes, like population fragmentation via biogeographic barriers, drive the generation of biodiversity (e.g., Gould and Lewontin, 1979; Gould, 2002). For example, the *Achatinella* snails of the Hawaiian Islands show great morphological variation in shell size, shape, and color but these traits have no obvious ecological association (Holland and Hadfield, 2007). Rather, shell morphology has evolved through random processes and species divergence is due to population fragmentation along the island's valleys, which are topographically complex (Holland and Hadfield, 2007). Similarly, the *Batrachoseps* salamanders in North America have split into several species, with some taxa being separated by over 10 million years, yet, little ecological and morphological differences exists among species (Wiens et al., 2007). Within *Batrachoseps*, species divergence is a function of colonization events into new habitat. However, *Batrachoseps* possess poor dispersal capabilities so that little gene flow occurs between new areas colonized and the ancestral home range (Wiens et al., 2007). In both the *Achatinella* and *Batrachoseps* lineages, it is clear that selection is not at work

but rather stochastic processes resulting in population fragmentation are creating new species with no correlation between morphology and ecology.

Some of the most compelling demonstrations of the power of natural selection are observed when repeated evolution of the same ecology-morphology-performance association occurs. For example, within the Galapagos finches, the sharp-beaked ground finch ecomorph has evolved three independent times. Beak morphology among these three species are highly convergent and used to feed on the same food type. Similarly, the *Anolis* lizards have independently evolved the same ecomorph several times so lineages that share the same ecology are morphologically more similar to each other than sister taxa that utilize different ecologies. In the case of biogeographic barriers, fragmentation leads to the divergence of species, but the resulting phenotypes may not change or they may vary randomly. Therefore, convergence, or in the extreme case, replicated adaptive evolution, is powerful evidence of the reality of ecological roles or "niches". While there is evidence for both adaptive and vicariant processes, what is less clear is how the processes interact to produce patterns of diversity.

The Asterophryinae and New Guinea as a Study System

The *Asterophryinae* frogs of New Guinea present an interesting system to study these questions as they are exceptionally diverse and live in one of the most geologically active parts of the world. The subfamily contains over 300 described species and it is believed that many more are yet to be discovered. Early taxonomists recognized that species vary widely in morphology in ways that seem to match their habitats, with frogs taking on similar shapes depending on whether they lived subterranean, ground, semi-aquatic, shrub, or tree habitats (Zweifel and Tyler, 1982), but no formal evolutionary or ecological studies have been performed. Therefore, the Asterophryine frogs present a model system to study how selection and vicariance interact to create diversity.

Prior work on the Asterophryinae has focused on resolving the taxonomic relationships, which has been fraught with difficulty (Burton, 1986; Burton and Zweifel, 1995; Frost et al., 2006; Günther, 2009; Kraus, 2013; Menzies and Tyler, 1977; Peloso et al., 2015; Zweifel, 1956, 2000; Zweifel and Allison, 1982; Zweifel and Parker, 1989). These difficulties were potentially exacerbated by the use of convergent morphological

traits to infer evolutionary relationships within the group leading to decades of taxonomic instability. Köhler and Günther (2008) were among the first to construct a molecular phylogeny for the Asterophryinae but the phylogeny was taxonomically undersampled, including only 40 taxa, and contained relatively little genetic data, making it difficult to test evolutionary hypotheses.

Here, I study the Asterophryinae of New Guinea, to determine how evolutionary processes interact to create biodiversity. I ask three questions: 1) What are the phylogenetic relationships of the clade and what role have geologic events played in promoting species diversification? 2) What is the role of natural selection? Is there an environment-morphology correlation? 3) Do these morphologies impart differential performance capabilities? By addressing these three questions, I can understand the dominant force creating diversity in the clade and discuss role of geology in the diversification of the *Asterophryinae* frogs. Furthermore, given the group's diversity, the *Asterophryinae* could become a model system for studying the interplay between adaptation and island biogeography in the creation of biodiversity.

CHAPTER 2. MOLECULAR PHYLOGENETICS AND DATING OF THE PROBLEMATIC NEW GUINEA MICROHYLID FROGS (AMPHIBIA: ANURA) REVEALS ELEVATED SPECIATION RATES AND NEED FOR TAXONOMIC RECLASSIFICATION

Abstract

Asterophryinae is a large monophyletic subfamily of Anurans containing over 300 species distributed across one of the world's most geologically active areas – New Guinea and its satellite islands, Australia and the Philippines. The tremendous ecological and morphological diversity of this clade, with apparent specializations for burrowing, terrestrial, semi-aquatic, and arboreal lifestyle, suggests an evolutionary process of adaptive radiation. Despite this spectacular diversity, this and many other questions of evolutionary processes have received little formal study because until now the phylogeny of this spececies-rich clade has remained uncertain. Here we reconstruct a phylogeny for Asterophryinae with greatly increased taxon and genetic sampling relative to prior studies. We use Maximum Likelihood and Bayesian Inference methods to produce the most robust and comprehensive phylogeny to date containing 155 species using 3 nuclear and 2 mitochondrial loci. We also perform a time calibration analysis to estimate the age of the clade. We find support for the monophyly of Asterophryinae as well as need for taxonomic reclassification of several genera. Furthermore, we find increased rates of speciation across the clade supporting the hypothesis of rapid radiation. Lastly, we found that adding taxa to the analysis produced more robust phylogenetic results over adding loci.

Introduction

The subfamily Asterophryinae is the largest part of one of the largest amphibian families in the world with nearly 300 described species of frogs (AmphibiaWeb; http://www.amphibiaweb.org/, accessed February, 2017). The majority of this diversity is centered in the Papuan region (comprising New Guinea and its satellite islands, the Admiralty and Bismark Archipelagos, and the Solomon Islands) and extends into northeastern Australia, the southern Philippines, and the eastern islands of the Sunda Shelf. Peloso et al. (2015) found asterophryine to be monophyletic and has most recently been organized into 21 genera. However, intergeneric relationships remain uncertain despite decades of study, hampering further evolutionary studies of this group.

In addition to its prolific numbers of species, Asterophryinae frogs are most notable for their high degrees of endemism and ecological diversity relative to the other four anuran families in the region. Taxonomists have identified six types of burrowing (fossorial), terrestrial, semi-aquatic, scansorial, and arboreal forms (Zweifel and Tyler, 1982) or putative "ecomorphs," indicating exceptional morphological disparity. While many questions remain unsettled in the Asterophryinae phylogeny, some of the ecomorphs appear in seemingly unrelated lineages (Köhler and Günther, 2008), suggesting repeated, independent evolution to novel lifestyles. Indeed, Asteroprhyinae is an excellent candiate adaptive radiation - a rapidly radiating lineage coupled with ecological diversification (Schluter, 2000).

The great ecological and species diversity may be linked in part to the geological history of New Guinea. The Papuan region lies at the junction of three active plates: the northward-moving Australian Plate, the west-northward-moving Pacific Plate the stable Eurasian Plate (Kroenke, 1996; Kroenke, 1998; Hall, 1998; Klootwijk et al., 2003), which have combined to move entire island-arc systems as well as shearing off and rearranging sections of continental land masses. These tectonic activities have resulted in a great expansion of land area as both island arc systems and offshore terranes have sequentially collided to form the large present-day New Guinea island and its smaller offshore islands. (Pigram and Davies, 1987; Klootwijk et al., 2003). In addition, these large-scale geological events, such as the docking of the Fold Belt, have given rise to the

high mountains traversing central New Guinea creating topographical complexity and further expanding ecological opportunity. It seems likely that Asterophryinae was the first anuran lineage to colonize New Guinea as the island is estimated to be approximately 30 MY old (Davies et al., 1996; Davies et al., 1997), consistent with the lower end of the 30-60 MY range estimated by other molecular phylogenetic studies on Asterophryinae, and it is the most widespread and diverse anuran lineage (Roelants et al., 2007; van Boxclaer et al., 2006; van der Meijden et al., 2007)

Taxonomic Problems

Many previous studies have used morphological characters to infer the Asteroprhyinae phylogeny, however, some of the character data contain insufficient information to resolve the phylogeny. For example, the clavicles and procoracoids have been lost several times independently and are thought to be homoplastic traits (Burton, 1986; Duellman and Trueb, 1986) and therefore not informative regarding evolutionary relationship. There is also great variability in the cranial elements (Burton, 1986), which may potentially lead to erroneous conclusions about species relationships. It is therefore not entirely surprising that homoplasy is common, yielding conflicting phylogenetic conclusions based on morphological data (Burton, 1986; Menzies, 2006; Zweifel, 1972, 2000). As a result, taxonomy of the Asterophryinae has been fluid throughout the 20th Century, increasing from nine genera (Parker, 1934) to 21 in the span of 70 years, with several genera variously synonymized, resurrected, or redefined (Burton, 1986; Burton and Zweifel, 1995; Frost et al., 2006; Günther, 2009; Kraus, 2013; Menzies and Tyler, 1977; Peloso et al., 2015; Zweifel, 1956, 2000; Zweifel and Allison, 1982; Zweifel and Parker, 1989).

Few genera have been explicitly defined on the basis of synapomorphies; it is possible that some groups are paraphyletic or polyphyletic (Köhler and Günther, 2008). Köhler and Günther (2008) provided the most complete asterophryine phylogeny to date but were only able to sample one-fifth of the total current species, lacked some genera and ecomorphs, and lacked high support values for some newly proposed relationships. Consequently, relationships among many asterophryine taxa remain uncertain.

All currently recognized asterophryine genera merit explicit testing for monophyly given the uncertain evidence often available from morphology. Most hypotheses of monophyly to have been implicit in current or prior taxonomies of asterophryines. These include testing the monophyly of each of the 21 recognized genera. Furthermore, the synonymization or division of taxa proposed by previous studies should be formally tested, including the following hypotheses: (a) the synonymy of *Albericus* + *Choerophryne* (Kraus, 2013; Peloso et al., 2015); (b) the synonymy of *Albericus* + *Aphantophryne* + *Cophixalus* + *Copiula* + *Choerophryne*; (c) the division of *Asterophrys* into *Asterophrys* and *Hylophorbus* (Zweifel, 1972); (d) the division of Sphenophryne into *Sphenophryne*, *Austrochaperina, Oxydactyla*, *Liophryne* (Zweifel, 2000); and (e) the synonymy of *Xenorhina* + *Xenobatrachus* (Frost et al., 2006). Köhler and Günther (2008) suggested that several of these hypotheses are probably false, but they lacked the degree of resolution and taxonomic sampling required to make firm conclusions. Because taxonomic revision should be based on robust resolution of phylogenetic relationships, more comprehensive testing of each group's monophyly is required to achieve a reliable taxonomy and to promote further evolutionary studies.

Here, we construct the most comprehensive and well-supported molecular phylogeny to date for the Asterophryinae clade in order to clarify higher taxonomic units and intergeneric relationships. We will explicitly test the hypothesis of monophyly of the asterophryine lineage and hypotheses (a)-(e) above. We use this phylogeny to infer the number of independent origins of each of the several ecomorphs. Furthermore, we perform a time-calibration analysis to estimate the age of the clade and explore whether major geological events may have facilitated diversification within this clade. We also perform an ancestral-reconstruction analysis to test whether lineages with the same ecology are closely related or have arisen independently. Lastly, we comment on challenges encountered during our reconstruction of the phylogeny, our solutions to them, and suggestions for further research to resolve uncertainties.

Material and Methods

Specimens and Genetic Sequencing

Our study included 155 species of Asterophryinae representing 21 proposed genera (Table 1S). We obtained liver samples from specimens housed at the Bishop Museum, Honolulu, Hawaii (BPBM), Museum of Vertebrate Zoology at Berkeley (UMZ), University of Michigan Museum of Zoology (UMMZ), Zoologisches Museum Berlin (ZMB), and University of Kansas Biodiversity Institute and Natural History Museum (KU; see appendix for specimen list), as well as liver tissue collected in the field by the authors. We rooted the phylogeny using 3 outgroup taxa that are thought to include the sister taxon and more distantly related lineages -- *Dyscophus antongilii, Scaphiophryne marmorata*, and *Platypelis grandis* (van der Meijden, 2007). We sequenced three unlinked nuclear loci and two mitochondrial loci: Seventh in Absentia (*SIA*), Brain Derived Neurotrophic Factor (*BDNF*), Sodium Calcium Exchange subunit-1 (*NXC-1*), Cytochrome oxidase *b* (*Cyt b*), and NADH dehydrogenase subunit 4 (*ND4*), resulting in a total of ~2800 base pairs of sequence data (primer details in Table 2.1). Originally, we amplified three additional loci (*Rag-1*, *Rag-2*, and *BMP2)* but we were not confident in these data because they had high discordance among sequences. To eliminate the possibility of including non-homologous non-coding duplications, these loci were not used in the analysis.

Table 2.1. Primer sequences for the nuclear loci SIA, BDNF, NXC-1 and the mitochondrial loci Cyt *b* and ND4.

Gene	Primer Sequence 5' – 3'	Base Pairs	Reference
SIA: For	TCGAGTGCCCCGTGTGYTTYGAYTA	~400	Bonacum et al., 2001
SIA: Rev	GAAGTGGAAGCCGAAGCAGSWYTGCATCAT	~400	Bonacum et al., 2001
BDNF: For	ACCATCCTTTTCCTTACTATGG	~600	van der Meijden et al., 2007
BDNF: Rev	CTATCTTCCCCTTTTAATGGTC	~600	van der Meijden et al., 2007
NXC-1: For	GACCTTGGTCCMAGNACCATT	~650	This study
NXC-1: Rev	TSACTGCTTTCCTTGCYTG	~650	This study
Cyt *b*: For	TADGCRAAWAGRAARTAYCAYTCNGG	~600	Kurabayashi (unpublished)
Cyt *b*: Rev	ACMGGHYTMTTYYTRGCHATRCAYTA	~600	Kurabayashi and Sumida, 2009
ND4: For	CACCTATGACTACCAAAAGCTCATGTAGAAGC	~600	Arévalo et al., 1994
ND4: Rev	TATTAGGAGATGTTCTCG	~600	This study

DNA sequencing followed standard protocols. Total DNA was extracted from liver tissue using a Qiagen DNA extraction kit. We performed bidirectional PCR amplification using 25 μL reactions containing 0.25 units of GOTaq DNA polymerase (Promega), <50 ng/μl genomic DNA, 0.5μmol of each primer, 15 nmol of dNTP, and PCR buffer. We used an initial denaturing period of 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, annealing temperatures for 30 s, and an extension period of 72°C for 60 s followed by a final extension period of 5 min. Annealing temperature is as follows for each locus: 62°C for *SIA*, 56°C for *BDNF*, 55°C for *NXC-1*, 57°C for *Cyt b* and 51.4° C for *ND4*. We cleaned the reactions with Exo-SAP and sequenced using Applied Biosystems BigDye terminator chemistry on an ABI 3730XL sequencer

following standard protocols. Sequencing was conducted at the University of Hawai`i at Manoa's Advanced Studies of Genomics, Proteomics and Bioinformatics facility.

Sequence alignment and phylogenetic analyses

We prepared the sequence data for analysis as a concatenated dataset as well as a locus-by-locus partitioned dataset. We read and manually edited each sequence using Sequencher v4.8 (Sequencher v4.8). We aligned the sequences using ClustalX using default parameters (Larkin et al., 2007). Alignments for the microhylid dataset were unambiguous for all loci, lacking insertion and deletion events.

Concatenated phylogenetic analyses

We chose model of sequence evolution using log-likelihood ratio and Akaike information criteria (AIC; Akaike, 1974) as implemented in jModelTest v2.1.3 (Guindon and Gascuel, 2003; Posada, 2008). We used the GTR+I+G model best-fit the concatenated data. We used this model to infer both the Maximum Likelihood (ML) trees and Bayesian Inference (BI) trees below. Bootstrap support under the ML analysis was assessed using 1000 nonparametric bootstrap replicates using RaxML v8.0 (Felsenstein, 1985; Stamatakis, 2014).

We used MrBayes v3.2.1 to infer Bayesian (BI) trees (Ronquist and Huelsenbeck, 2003). Six Metropolis-coupled Markov-chain Monte-Carlo (MCMC) analyses were run for 20,000,000 generations, sampling every 100 generations with a burn-in of 25%. The average standard deviation of split frequencies was less than 0.01 indicating convergence on a single tree. A 50% majority-rule consensus of post burn-in trees was constructed to summarize posterior probabilities for each branch. Tracer v1.5 was used to ensure that proper mixing occurred by the MCMC runs (Drummond and Rambaut, 2007). Phylogenies were visualized using FigTree v1.3.1 (Rambaut and Drummond, 2009).

Partitioned time-calibrated analysis

We partitioned the data set by locus and used jModelTest to identify the following best-fitting models: *SIA*: HKY; *BDNF*: GTR; *NXC-1*: GTR+I+G; *Cyt b*: GTR+I+G; and *ND4*: HKY. We used BEAST to simultaneously estimate phylogenetic relationships and

nodal ages. The age estimates were calibrated using prior age distributions for specified nodes ('anchor points') that are associated with geological events dated with independent information (Table 2.2). The geological events represent the maximum age of a clade, under the assumption that amphibians cannot readily cross marine environments, and parametric distributions were modeled around these anchoring points (Ho et al., 2015). Calibration dates were modeled with a lognormal distribution, where 95% of the prior weight fell within the geological interval of the event. An uncorrelated, lognormal, relaxed-clock model with a Yule prior were used in the model that ran 20 000 000 generations sampling every 1000th generation with a 25% burn-in. Post burn-in trees were combined, and a maximum clade credibility tree was computed.

We chose a model-based approach for our phylogenetic reconstruction over maximum parsimony and other distance methods because model based approaches tend to be statistically more consistent (Chang, 1996a; Rogers, 1997) and in the case of BI, we can determine convergence on a single topology.

Table 2.2. Geological events used as calibration points used to anchor phylogeny

Node	Age (MY)	Geological Event	Daughter Taxa	Reference
A	6 ± 0.5	Woodlark Island Rossel Island	*Cophixalus clapporum* *Cophixalus cupricarenus*	Baldwin et al. 2012
B	4 ± 0.5	Sudest Island Rossel Island	*Mantophryne axanthogaster* *Mantophryne louisiadensis*	Hill et al. 1992
C	4 ± 0.5	Misima Island Sudest Island	*Hylophorbus sp. 8* *Hylophorbus extimus*	Hill et al. 1992
D	5 ± 1	Misima Island Woodlark Island	*Copiula oxyrhina* *Copiula sp. 5*	Baldwin et al. 2012
E	6 ± 0.5	Woodlark Island Misima Island	*Barygenys apodasta* *Barygenys exul*	Baldwin et al. 2012

Ancestral reconstruction and diversification analyses

The following ecomorph categories have been recognized based on the perch locations where frogs are most commonly found: arboreal, scansorial, terrestrial, fossorial, and semi-aquatic (Zweifel and Tyler, 1982). The distinction between the two climbing specialists, arboreal and scansorial, was first made by Inger (1954), with scansorial species using shrubs and low-to-the-ground perches while arboreal species are found on tall trees and tree-holes. We conducted an ancestral-state reconstruction analysis to study ecomorph evolution, with particular focus on the minimum number of transition events between ecomorph categories. We used both maximum likelihood and parsimony to infer the ancestral evolution of ecomorphs on the recovered Bayesian tree. Ecomorph information from the tips of the time-calibrated phylogeny was used to infer the states at internal nodes. The maximum-likelihood analysis was performed in R using the package Ape (Paradis et al., 2004). Uncertainty at each node was calculated in the maximum-likelihood analysis, and those nodes with two or more possible reconstructions are labeled with pie charts indicating the strength of support for each possibility.

We estimated the average rate of diversification across the tree. To visualize the rate of lineage accumulation over time, we constructed a lineage-through-time (LTT) plot for the BI tree (Nee et al., 1992). The γ-statistic reports the mean diversification rate (Pybus and Harvey, 2000). Our null hypothesis was constant diversification rate through time ($\gamma=0$). An increasing diversification rate ($\gamma>0$) would show a rapid accumulation of lineages yielding a concave LTT plot, whereas a decreasing diversification rate ($\gamma< 0$) would result in a convex LTT. We note that measures of lineage accumulation do not distinguish speciation from extinction rates. We used R statistical programming environment (R core team, 2015) to perform all diversification analyses using the packages Ape (Paradis et al., 2004), Geiger (Harmon et al., 2008), and Laser (Rabosky and Schliep, 2013) on a phylogenetic tree with branch lengths.

Results

Phylogenetic analysis

We retrieve Asterophryinae as monophyletic, with a topology that is robust to analytical method, being nearly identical whether analyzed by ML or either BI methods, and whether the DNA sequence data is concatenated or partitioned by locus. The few minor disagreements between the inferred tree topologies occurred at the terminal nodes. In the ML analysis, *Oreophryne brachypus* is the sister taxon to *Oreophryne geislerorum* and *Oreophryne species* 1, whereas in the BI analysis, the three form a supported trifurcation. Similarly, the clade composed of *O. species* 3, *O. species* 2, *O. lemur*, and *O. species* 4 form a resolved clade in the ML analysis but cluster in an unresolved polytomy on the BI tree. The two remaining discrepancies occur in the *Hylophorbus* clade, where *H. species* 10 and *H. species* 9 form a clade that is the sister taxon to *H. species* 11 in the ML analysis, whereas they form a trifurcation in the BI analysis. Lastly, the ML analysis shows that *H. proekes*, *H. species* 3 and *H. species* 2 form a trifurcation, whereas the BI analysis shows that *H. species* 2 forms a clade with *H. proekes*, which together form the sister taxon to *H. species* 3. Again the vast majority of the topology is identical between methodologies.

Monophyly. Five of the 21 recognized genera have strong support for their monophyly in both the ML and BI analyses: *Callulops*, *Hylophorbus*, *Mantophryne, Xenorhina*, and *Barygenys*. The remaining 14 genera are not monophyletic and require varying degrees of reclassification.

Rejection of monophyly. We do not find support for the monophyly of genus *Oreophryne*. At the same time, we cannot reject monophyly for Oreophryne. There is no statistical support for the branches that connect the three *Oreophryne* groups therefore, we do not know the placement of this genus. We do find that *Aphantoprhyne* is nested within *Oreoprhyne-3* and synonymy may be indicated. Similarly, we cannot accept nor reject the monophyly of *Austrochaperina* or *Copiula* as there is no statistical support to the branches connecting the species.

Paraphyly. Eleven genera are paraphyletic but can be brought into monophyly by collapsing genera. The genera *Metamagnusia* and *Asterophrys* are paraphyletic, but can form a clade if synonymized into *Asterophrys* along with *Pseudocallulops*. Similarly, *Liophryne* is paraphyletic but can be made monophyletic if synonymized with the genera nested within *Liophryne*: *Oxydactyla* and the monotypic genera *Genyophryne* and

Sphenophryne. We also find that *Copiula* is paraphyletic with one species nested within *Cophixalus* with strong statistical support and separated by several strongly-supported nodes from three additional species grouping elsewhere on the tree. *Choerophryne* is also paraphyletic as a *Paedophryne* species is nested within it, also making *Paedophryne* paraphyletic because a pair of *Paedophryne* are distantly related to this lone species. The predominantly terrestrial genus *Cophixalus* is paraphyletic as one species of *Copiula* is nested within the lineage.

Dating and diversification analysis

Calibration of the molecular phylogeny with geological dates (Table 2.2) indicate that Asterophryinae is roughly 28 MY old (Figure 2.2). There is evidence of increased lineage accumulation between 15-17 MYA, 6-11 MYA, and as recent as 2 MYA (Figure 2.3). The nodal heights and 95% highest posterior density (HPD) intervals inferred with BEAST are graphically represented in Figure 2.2; date estimates for labeled nodes are reported in Table 2.3.

The concave shape of the LTT plot indicates that the Papuan microhylids experienced rapid lineage accumulation (Figure 2.3). Over the history included within the phylogeny, lineage accumulation occurred at significantly higher rates than expected under the null model of constant diversification through time (high diversification rate: γ=3.83; p-value=0.05).

Table 2.3. Estimates of node age at calibration points indicated on Figure 2.2 based on molecular dating analysis.

Node of interest	Description	BEAST analysis, mean nodal ages in MY (CI[a])
-	Origin of Asterophryinae	27.65 (16.88-39.29)
A	Split between *Cophixalus clapporum* and *Cophixalus cupricarenus*	5.40 (3.41-7.33)
B	Split between *Mantophryne axanthogaster* and *Mantophryne louisiadensis*	3.76 (2.24-5.32)
C	Split between *Hylophorbus sp. 8* and *Hylophorbus extimus*	3.45 (2.04-4.89)
D	Split between *Copiula oxyrhina* and *Copiula sp. 5*	3.99 (2.42-5.60)
E	Split between *Barygenys apodasta* and *Barygenys exul*	6.24 (4.60-7.94)

[a] Confidence Intervals (CI) for BEAST analysis refers to the 95% HPD interval.

Ancestral reconstruction analysis

There is evidence for at least 22 ecological shifts over the course of Papuan microhylid evolution (Figure 2.1). The inferred shifts did not differ between the parsimony and maximum likelihood analyses, and therefore, only results from the maximum likelihood analysis will be discussed here. All five ecomorphs evolved early in the clade's history, ~17 MYA, with prolific lineage accumulation occurring subsequently. There is a general pattern of niche conservatism within genus, but there are also subsequent independent shifts in ecomorph category for each ecomorph type. The base of the tree is inferred to have been a terrestrial ecomorph (Figure 2.1). Alternative ancestral reconstructions were also considered. Deeper nodes could alternatively be coded as fossorial (red) but this would incur an additional four state shifts

along the tree. If the deepest node is coded as arboreal (green) it would imply one additional shift.

Discussion

Systematic implications

For decades, the taxonomy of this large group was based on the presence or absence of morphological characters. These were often functional characters related to lifestyle, and therefore could be homoplastic. Later attempts at phylogenetic reconstruction used DNA evidence but suffered from low levels of taxonomic sampling. We discuss below our taxonomic findings based on inclusion of more than half of the known diversity of Asterophryinae with multiple nuclear and mitochondrial loci. We also summarize our taxonomic suggestions in the supplemental material (2S).

Zweifel (1971, 1972) proposed dividing Papuan microhylids into the two traditional subfamilies Genyophryninae (contemporarily: *Austrochaperina*, *Aphantophryne*, *Choerophryne*, *Liophryne*, *Oreophryne*, *Oxydactyla*, and *Sphenophryne*) and *sensu* Zweifel Asterophryinae (contemporarily: *Asterophrys*, *Barygenys*, *Callulops*, *Hylophorbus*, *Pherohapsis*, *Mantophryne*, *Xenobatrachus* and *Xenorhina*). This separation was based on morphological elements for example, the complete pectoral girdle and symphygnathic maxillary. Instead, the phylogeny provides support for a single Asterophryinae clade, and elimination of the Genyophryninae subfamily nomenclature, as already followed by Frost et al. (2006). We have also shown here that a single genus can exhibit a variety of lifestyles and adaptations that make traits related to ecomorph classification homoplastic. For example, Zweifel (1972) said that a symphygnathine state of the maxillary is diagnostic of Asterophryinae, but Köhler and Günther (2008) found that it had been secondarily lost in *Hylophorbus*. Furthermore, presence of a complete pectoral girdle is considered plesiomorphic, but many unrelated Asterophryinae lineages do not have a complete pectoral girdle (Duellman and Trueb, 1986; Burton, 1986), indicating that the loss of this trait occurred several times independently across the lineage in *Austrochaperina, Albericus, Barygenys*, *Callulops, Choerophryne*, *Cophixalus*, *Copiula*, *Hylophorbus, Xenorhina* (Burton, 1986; Zweifel, 1972; Menzies, 2006), and

should not be used as a character for phylogenetic reconstruction as it contains no information on relationship. We discuss our findings relative to the monophyly of genera below, in order of the phylogeny presented in Figure 2.1.

Our analysis indicates synonymy of the two genera *Albericus* (Burton and Zweifel, 1995) and *Choerophryne* (Van Kampen, 1914), which comprise 31 species that are scansorial as already followed by Peloso et al. (2015). This genus forms a clade with strong support as proposed by Burton and Zweifel (1995; hypothesis a; see section 1.1) on the basis of morphological synapomorphies. These two genera were distinguished on the basis of the alary process of the premaxilla, oriented dorsally in *Albericus* and anteriorly in *Choerophryne* (Burton and Zweifel, 1995). Kraus (2013) described a new species of *Choerophryne* with an intermediate alary process providing morphological corroboration of the molecular results here, namely of a single genus with a continuum of alary-process orientation in this character. Therefore, our results agree with the synonymization of *Albericus* with *Choerophryne*. Furthermore, we did not find that *Albericus* and *Choerophryne* are closely related to *Aphantophryne*, *Cophixalus*, or *Copiula*, (hypothesis b; see section 1.1). The defining feature for this hypothesis was the loss of clavicles and procoracoids. Our analysis indicates that these losses occurred multiple times independently.

Aphantophryne Fry, 1917, is a terrestrial clade of three described species nested within the *Oreophryne*-3 clade. Although *Aphantophryne* is monophyletic by our analysis, recognizing it as a separate clade would make *Oreophryne*-3 paraphyletic. Therefore, we suggest combining *Aphantophryne* and *Oreophryne*-3 to form a single monophyletic genus under the *Oreophryne genus*. Rittmeyer et al. (2012) and Pyron and Wiens (2011) found that *Aphantophryne* was the sister taxon to *Cophixalus* not *Oreophryne*, but their sampling was limited. In addition, *Aphantophryne pansa* is represented here by four lineages that are separated by roughly 5 MY. This is suggestive of a species complex rather than a single widespread species, and needs further study.

Asterophrys Tschudi, 1838 is a genus comprising two species and was previously thought to be closely related to *Callulops*. We find that it forms a clade with *Metamagnusia* (Günther, 2009) and *Pseudocallulops* (Günther, 2009). This was also found by Peloso et al. (2015) and Rittmeyer et al (2012). Furthermore, we find support

for the hypothesis (c) proposed by Zweifel (1972), which split *Asterophrys* from *Hylophorbus*. We do not find support for Köhler and Günther's (2008) and Pyron and Wiens' (2011) finding that *Asterophrys* is closely related to *Callulops* and *Xenorhina*. Therefore, we recommend synonymization of *Megamagnusia* and *Pseudocallulops* into *Asterophrys* to create a clade.

Austrochaperina Fry, 1912 is a predominantly terrestrial group that also contains two semi-aquatic species. We cannot accept nor reject the monophyly of Austrochaperina, and therefore this group needs further investigation. We did find some evidence for two clades of Austrochaperina but no support for joining these two smaller clades to form a monophyletic grouping. In addition it is unclear where Austrochaperina palmipes (the semi-aquatic species) should be placed within the Asterophryine tree. Pyron and Wiens (2011) and Rittmeyer et al (2012) found *Copiula* and *Austrochaperina* to be interdigitated phylogenetically; we find only one species of *Copiula* to be nested within some *Austrochaperina*. Zweifel (2000) proposed splitting *Austrochaperina*, *Sphenophryne*, *Oxydactyla*, and *Liophryne*. We find support for synonymization of *Spenophryne*, *Oxydactyla*, and Liophyrne (see below).

Barygenys Parker, 1936 is a small fossorial genus of nine species and was thought to be closely related to *Cophixalus* by Sumida et al. (2000), Frost et al (2006), and Köhler and Günther (2008). Peloso et al. (2015) placed *Barygenys* as the sister taxon to *Genyophryne* whereas Rittmeyer et al. (2012) found it to be the sister taxon to *Paedophryne*. We found support for the monophyly of Barygenys, however, we found no support for any of these higher-order hypotheses making its placement within Asterophryinae still unclear.

Callulops Boulenger, 1888 is a fossorial group thought to be non-monophyletic by Köhler and Günther (2008), who found some species of *Callulops* closely related to *Asterophrys*, with other species more closely related to *Hylophorbus*. Pyron and Wiens (2011) found *Callulops* to be non-monophyletic and the sister taxon to *Xenorhina* and *Asterophrys*. In contrast to these previous studies, we find that *Callulops* forms a strongly supported clade that is the sister taxon to *Mantophryne* and *Hylophorbus* which also supports the relationships obtained by Rittmeyer et al. (2012) and Peloso et al. (2015) for these three genera.

Cophixalus Boettger, 1892 forms the sister taxon to a clade comprising all other genera of Asterophryinae. Köhler and Günther (2008), Pyron and Wiens (2011), and Rittmeyer et al. (2012) retrieved the genus as non-monophyletic, which variously grouped with different genera depending on the study. We find that *Cophixalus* forms a clade pending inclusion of *Copiula sp. 1*. It is important to mention that previous studies have found *Cophixalus* to be polyphyletic, but our results do not support this finding.

Copiula Méhely, 1901, was found to be non-monophyletic by Köhler and Günther (2008) with some species falling within *Austrochaperina* and other species forming the sister taxon to the *Austrochaperina* clade. Other studies have found an interdigitated pattern for the two genera *Copiula* and *Austrochaperina* (Pyron and Wiens,2011; Rittmeyer et al., 2012; and Peloso et al., 2015), which may support their inclusion in a single genus, but this needs further study. We find a similar pattern in that the single species *Copiula tyleri* falls within *Austrochaperina*. Beyond this, the relationship between *Copiula* and *Austrochaperina* is not clear as there is no statistical support at the base of either of these genera. Lastly, we do not know the placement of *Copiula minor* and *C. fistulans* within the Asterophryinae due to the lack of support for the branches that relates these two species to all other *Copiula* species. We note that *Copiula* remains a major problem for our tree. These are species that are morphologically homogeneous and can be difficult to distinguish from one another, yet we have these very similar animals partitioned into unrelated parts of the tree.

Genyophryne thomsoni is a monotypic genus that was found to be closely related to *Liophryne* in previous studies (Köhler and Günther, 2008; Pyron Wiens, 2011), and our results also support this finding. In fact, *Genyophryne* is nested within *Liophryne* along with the genera *Oxydactlya* and *Sphenophryne*. This result is not supported by Peloso et al. (2015) as they found *Genyophryne* to be more closely related to *Barygenys*. We suggest synonymizing *Genyophryne, Oxydactlya* and *Liophryne* into *Sphenophryne* to form a clade, but we caution that this also needs further study.

Hylophorbus Macleay, 1878 was separated from *Asterophrys* by Zweifel (1972). Our results support the split between these two genera. We find that *Hylophorbus* is the sister taxon to *Mantophryne* which is also supported by Rittmeyer et al. (2012), Oliver et al. (2013), and Peloso et al. (2015).

Mantophryne Burton, 1986 has been absent from other studies, with the exception of Oliver et al. (2013), and its placement in the broader asterophryine phylogeny was previously unknown (Köhler and Günther, 2008; Pyron and Wiens, 2011). We find that *Mantophryne* forms a well-supported clade sister to *Hylophorbus*. Furthermore, we also find support to the transferring of *Pherohapsis menziesi* into *Mantophryne* (now *Mantophryne menziesi*).

Oreophryne Boettger, 1895 is the most geographically widespread group in Asterophryinae whose distribution ranges from the Philippines and Bali down to the satellite islands of PNG (Kraus, 2013). We find evidence for at least three clades of *Oreophryne* depicted in Figure 2.1. However, we have no support for uniting these three clades nor for their independence, thus it is unclear whether Oreophryne is a single monophyletic group or polyphyletic. Although there are a few terrestrial alpine lineages (not sampled in this study), most *Oreophryne* species are arboreal and possess morphologies beneficial for climbing such as enlarged toe pads and elongated limbs. Previous workers used these shared morphologies as proxies for close evolutionary relationship, however, they may instead result from convergent evolution to shared lifestyles. Indeed, Zweifel et al. (2005) has suggested that these characters may have arisen by homoplasy and the genus may be polyphyletic. Further geographical sampling of the wide-spread *Oreophryne* may increase phylogenetic resolution by providing more data to discern ancient splits, and we recommend this as a high priority for future study.

Paedophryne Kraus, 2010 is a genus that includes one of the smallest frogs in the world (Rittmeyer et al., 2012). *Paedophryne* was erected based on the reduced number of presacral vertebrae, reduction of phalanges, tongue shape, lack of digital discs, and configuration of three different muscles. Many of these traits are related to miniaturization, and may have arisen independently in different lineages undergoing size reduction. We sampled three species of *Paedophryne*, two of which we place as the sister taxon to *Cophixalus*. A single species, *Paedophryne swiftorum*, was found to be more closely related to *Albericus* and *Choerophryne*, with moderate support. Rittmeyer et al. (2012) found moderate support for the genus' monophyly but no other phylogenetic studies address their taxonomy and with limited samples more work would be beneficial.

Xenorhina Peters, 1683 is a predominantly fossorial lineage. Frost et al (2006) synonymized *Xenobatrachus* into *Xenorhina* based on a molecular phylogeny (hypothesis e), a result which is now well-supported by several studies (de Sa et al. 2012; Peloso et al. 2015; this study; Figure 2.1 shows only the synonymized clade). What remained uncertain was the sister taxon to *Xenorhina* We clarified the sister group relationship, showing that *Xenorhina* is the sister taxon to *Callulops, Mantophryne*, and *Hylophorbus*.

Dating and Biogeography

New Guinea is in an unusually active geological region of the world. At the junction of 4 tectonic plates, the island has been formed by offshore island arcs and portions of continents are colliding together resulting in the formation of a larger island (called accretion events). As these have happened sequentially over millions of years, the landmass of New Guinea has grown through time. In addition, these accretion events created uplift which gave rise to the Central Mountains of New Guinea resulting in topographical complexity. These processes created new complex habitat and microhabitat providing ecological opportunity for those lineages that first colonized the island. It is not clear whether the land masses arrived with fauna at the time that they amalgamated onto New Guinea island, nevertheless, the several lines of circumstantial evidence suggests that asterophryines may have been the first anuran lineage to colonize the island. The age of the Asterophryinae clade, ~28 MY, implies that the group may have originated on the Australian Craton, the oldest terrane of New Guinea.

Asterophrines are also the most geographically-widespread and most species-rich anuran lineage. By approximately 20 MYA, all major ecological types had evolved, coinciding with the amalgamation of the Fold Belt to the Australian Craton (Pigram and Davies, 1987). This collision event gave rise to the central mountain range of New Guinea creating an altitudinal gradient that presumably led to the formation of new niches that the asterophryine could exploit. Other studies have shown that complex topography can lead to more isolated populations leading to higher speciation rates and endemism (Brown, 2001; Kessler, 2002). Furthermore, we also see an increased in lineage accumulation rates between 15-17 MYA and 6-11 MYA (Figure 2.2), which coincides with the date range of the amalgamation of the East Papuan Composite Terrane

(EPCT) and the accretion of terranes along northern NG as well as the Vogelkop Composite Terrane that forms the north-west section of NG. It is important to note that the dates of the geological events are actively being studied and vary between researchers. For example, Cloos et al. (2005) found that New Guinea began to form ~12 MYA and not 40 MYA. Despite the dates varying, the sequence of geological events are agreed upon therefore, many of our ecological hypotheses still stand.

The relationship between area and species numbers in *Anolis* lizards has been reviewed by Losos and Schluter (2000), who showed that the rate of species formation increases as island area increases. Here we have not only areal increase but also increase in habitat complexity. Future studies should examine the interaction between areal expansion and the increasing topographic complexity on the biota.

Our study does have limitations which we note here. For example, geographically, a majority of our sampling comes from EPCT with no lineages from the Central Mountains of New Guinea. These lineages remain largely undersampled due to the difficulty in fieldwork. Furthermore, It is important to note that the 95% highest posterior density (HPD) is large at the base of the phylogeny. This is in common with many other studies is a byproduct of lack of information at the base. Our calibration points occur closer to the terminal tips of the tree, increasing the certainty of the timing of those points, but we have no external information at the base, resulting in high uncertainty in dates at deep branches.

Ecomorphology

It is interesting to note that major ecological transitions occured early in the asterophryine history. Among the first steps in Asterophryine evolution is the diversification into disparate lifestyles. This does indeed correspond with current theory for adaptive radiations, with ecological opportunity giving rise to a period of expansive niche diversification, followed by much longer periods of niche stability (Schluter, 2000). Once the ecomorphs arose, there was prolific lineage accumulation. About half of the genera demonstrate ecological niche conservatism, with the other half showing various degrees of further ecological diversification (Figure 2.1). Over hundreds of speciation events, at least 22 ecomorphological shifts have occurred. Transitions seem to have

directionality, and have a somewhat predictable pattern. For example, scansoriality has evolved eight times throughout the phylogeny, each time from a terrestrial ancestor, with the exception of *Sphenophryne cornuta*, which evolved from a fossorial ancestor. The genus *Choerophryne* contains only scansorial ecologies, whereas scansoriality has evolved in the genus *Cophixalus* genus four independent times. Scansoriality has evolved once within *Liophryne* and once within *Mantophryne*. The semi-aquatic ecomorph only had evolved three times (two were sampled here), across distant regions of the phylogeny, twice within *Cophixalus* and once in *Austrochaperina*.

Arboreality has also evolved independently six times along the phylogeny. It has evolved in the genus *Oreophryne*, which may or may not be monophyletic, and twice within the non-monophyletic genus *Metamagnusia*. A somewhat peculiar lineage, *Xenorhina arboricola*, is an arboreal species that is nested within a completely fossorial clade. This pattern is not seen anywhere else in the phylogeny. However, this "arboreal" lineage lives burrowed in soil that accumulates in arboreal staghorn ferns, which is interesting in that the burrowing aspect is preserved, but whether it has new climbing adaptations is unknown.

The fossorial lifestyle has evolved six independent times. Three of these shifts evolved in the ancestor of the genera *Callulops*, *Xenorhina*, and *Barygenys*, all of which are predominantly fossorial. It also arose once in the clade comprising *Liophryne*, *Genyophryne*, *Oxydactyla* and *Sphenophryne*. In an unusual display of diversity, this clade contains three different ecologies including terrestrial, scansorial and fossorial ecologies. Fossoriality also evolved independently in *Pseudocallulops* and two species of *Copiula*

In all, eight unique types of transitions (out of 20 possible) were noted with the terrestrial ecology being the ancestor for the majority of them. The terrestrial ecomorph thus appears to be the "hub" ecomorph giving rise to more specialized forms, and in turn, for several of the specialist forms to revert back. There is surprisingly no evidence for evolutionary transition between the specialist types, with the exception of *Xenorhina arboricola* which is arboreal and nested within a fossorial clade. It would be interesting to explore whether there are constraints between specialist transitions and what forms of morphological evolution are required to change lifestyle.

There appears to be directionality to ecomorph transitions, with the most common transitions occurring from terrestrial ancestors to the other ecomorphs, having occurred 13/22 times. There is little indication that the reverse sequence has occurred. The only exception being *Aphantophryne* secondarily evolving terrestriality from arboreal ancestors.

Improving resolution in rapidly speciating lineages

Difficult-to-resolve phylogenies seem to be a hallmark of adaptive radiation. This is because by definition large evolutionary changes are occurring in often small windows of time where many species are diversifying. This rapid speciation leads to incomplete lineage sorting resulting in branches with no statistical support (Jackman et al. 1999). There are two prevalent strategies to solve this problem. Lamichhaney et al. (2015) found that they were able to resolve the Darwin's finches phylogeny by massively increasing genetic data (sequencing the entire genome), but we note that this particular clade is small and therefore the only option is to increase genetic sampling. For a large group such as ours, this strategy may not currently be cost-effective or practical in terms of the required data analysis. Other studies have shown that adding taxa is more beneficial over adding loci (Rannala et al., 1998; Zwickl and Hillis, 2002; Heath et al., 2008; Huang and Knowles, 2016).

We attempted both strategies. We first selected a limited number of taxa (11 taxa likely to span the phylogeny) and doubled our genetic loci from 5 to 10. Both the initial loci and additional loci were carefully chosen to span a range of mutation rates. However, we did not obtain convergence on a stable topology, and instead found major shifts in topology as genetic data were added (each of which were strongly supported but incompatible). Alternatively, we found that adding more taxa, particularly from large, widely distributed clades (i.e., breaking up potentially long branches) produced the most stable phylogeny over increasing genetic sequences. Our earlier phylogeny contained 75 OTU's with 59% of the nodes supported. Increasing to 122 OTU's resulted in 65% of nodes having support. Here, we present a phylogeny with 155 OTU's with 70% supported nodes. It is clear that there is a positive relationship between the addition of taxa and the statistical support for the tree, therefore, we recommend judicious addition

of species, particularly if the problematic phylogeny contains widespread, potentially basal clades and with an aim to break up long branches.

Our findings are consistent with Heath et al. (2008), who found that by adding taxa, homoplastic characters are distributed across the tree lessening the effects of long-branch attraction and reducing the recovery of erroneous relationships. Although long-branch attraction was first described in the context of parsimony (Felsenstein, 1985), a review by Bergsten (2005) showed that even model-based approaches such as ML and BI suffer from this problem too. Furthermore, we stress the importance of strategically adding taxa to break-up the long branches while simultaneously not introducing additional long branches (Heath et al., 2008). In our example, the addition of loci to a phylogenetic problem containing only distantly related taxa would do nothing to break up long branches. Alternatively, adding greater representation of basal taxa, especially geographically widespread clades have the potential to do so.

Conclusions

We produced a robust phylogeny for the large and diverse Asterophryinae using 3 nuclear and 2 mitochondrial genes on 155 taxa. We found that the current taxonomy for the clade may require some revision as several genera were based on characters that reflected lifestyle. Unsurprisingly, these few characters used in early taxonomic work were found to be evolutionarily labile, and furthermore, some of these characters were lost as opposed to gained. In essence, the characters were not vetted for establishing synapomorphy. Given our new phylogeny, calibrated with dates from known geological events, we were able to construct hypotheses regarding the importance of early ecological diversification and for the role of biogeographic events in promoting speciation. Importantly, whereas most other adaptive radiations involve geographic isolation between species, here we have one of the few examples of island amalgamation, where two or more landmasses collide, potentially bringing faunas together. Therefore, In addition to the well-known adaptive radiations such as the Caribbean *Anolis* lizards, the African cichild fishes, and the Hawaiian silverswords, the Papuan Asterophryine provides another intriguing example involving both ecological diversification and biogeography.

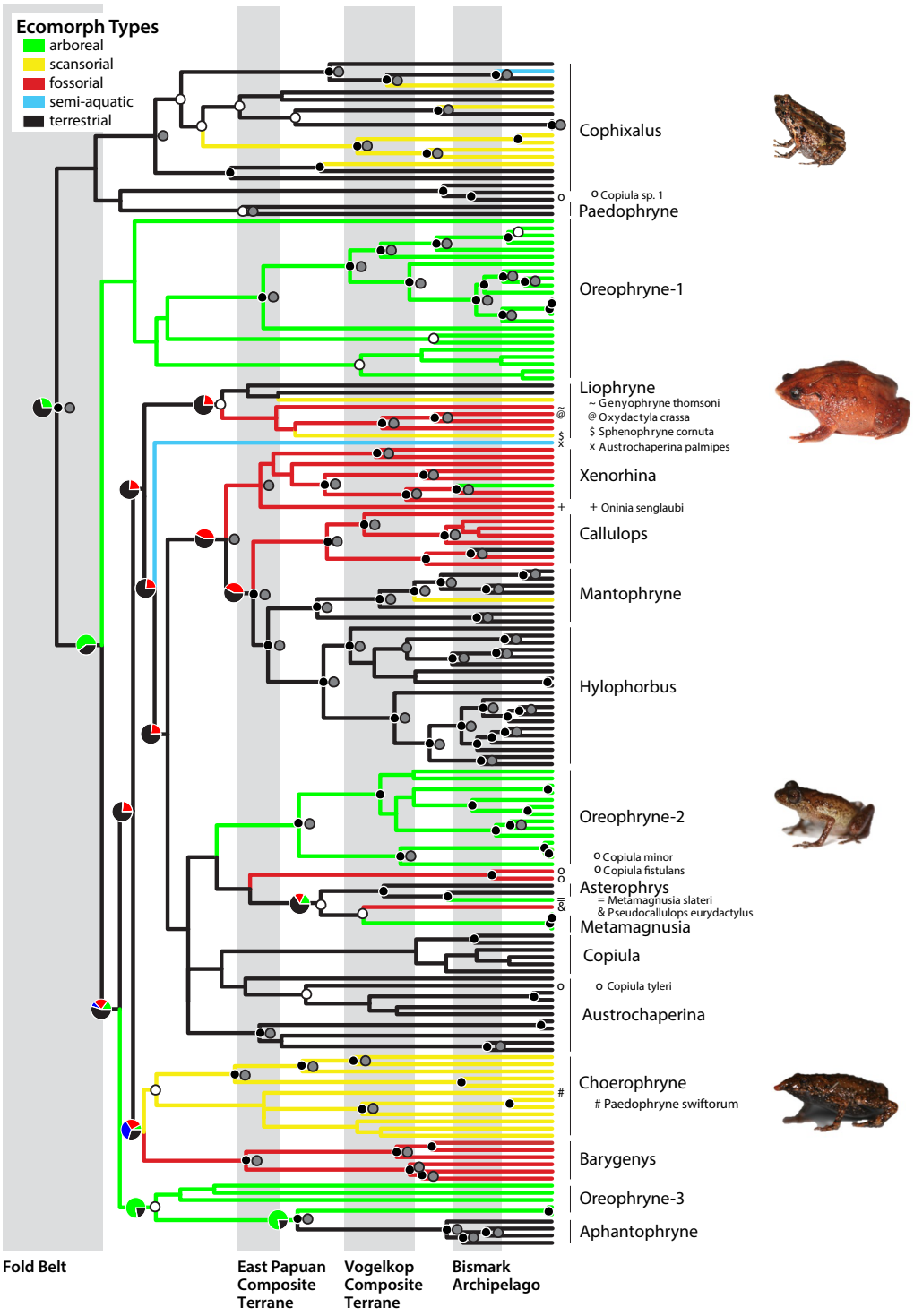

Ecomorph Types
arboreal
scansorial
fossorial
semi-aquatic
terrestrial
Cophixalus
o Copiula sp. 1
Paedophryne
Oreophryne-1
Liophryne
~ Genyophryne thomsoni
@ Oxydactyla crassa
$ Sphenophryne cornuta
x Austrochaperina palmipes
Xenorhina
+ Oninia senglaubi
Callulops
Mantophryne
Hylophorbus
Oreophryne-2
o Copiula minor
o Copiula fistulans
Asterophrys
= Metamagnusia slateri
& Pseudocallulops eurydactylus
Metamagnusia
Copiula
o Copiula tyleri
Austrochaperina
Choerophryne
Paedophryne swiftorum
Barygenys
Oreophryne-3
Aphantophryne
Fold Belt
East Papuan Composite Terrane
Vogelkop Composite Terrane
Bismark Archipelago

Figure 2.1. Molecular phylogeny of the Asterophryine clade with nodal support and the evolution of ecomorphs. Nodal support is indicated for all three phylogenetic reconstruction methods: dark circles indicate "highly supported" nodes from both MrBayes (>0.95 Bayesian posterior probability) as well as RaxML (ML bootstrap support >70%). while open circles represent "moderately supported" nodes (Bayesian posterior probabilities >0.90). Gray circles represent posterior probabilities for the BEAST analysis >0.95. No symbol on a node represents no statistical support for that split. Also shown is the ancestral-state-reconstruction analysis for the pattern of ecological evolution for Asterophryinae lineage. The colors correspond to their ecomorph type where red = forssorial, yellow = terrestrial, blue = scansorial, green = arboreal, and light blue = semi-aquatic. Nodes where multiple ecomorph types are possible are indicated by pie charts representing the level support for each possibility. Vertical gray bars indicate the timing of geological events. Pictures, from top to bottom, are *Cophixalus variabilis*, *Genyophryne thomsoni*, *Mantophryne lateralis*, *Oreophryne sp.*, and *Choerophryne sp.*

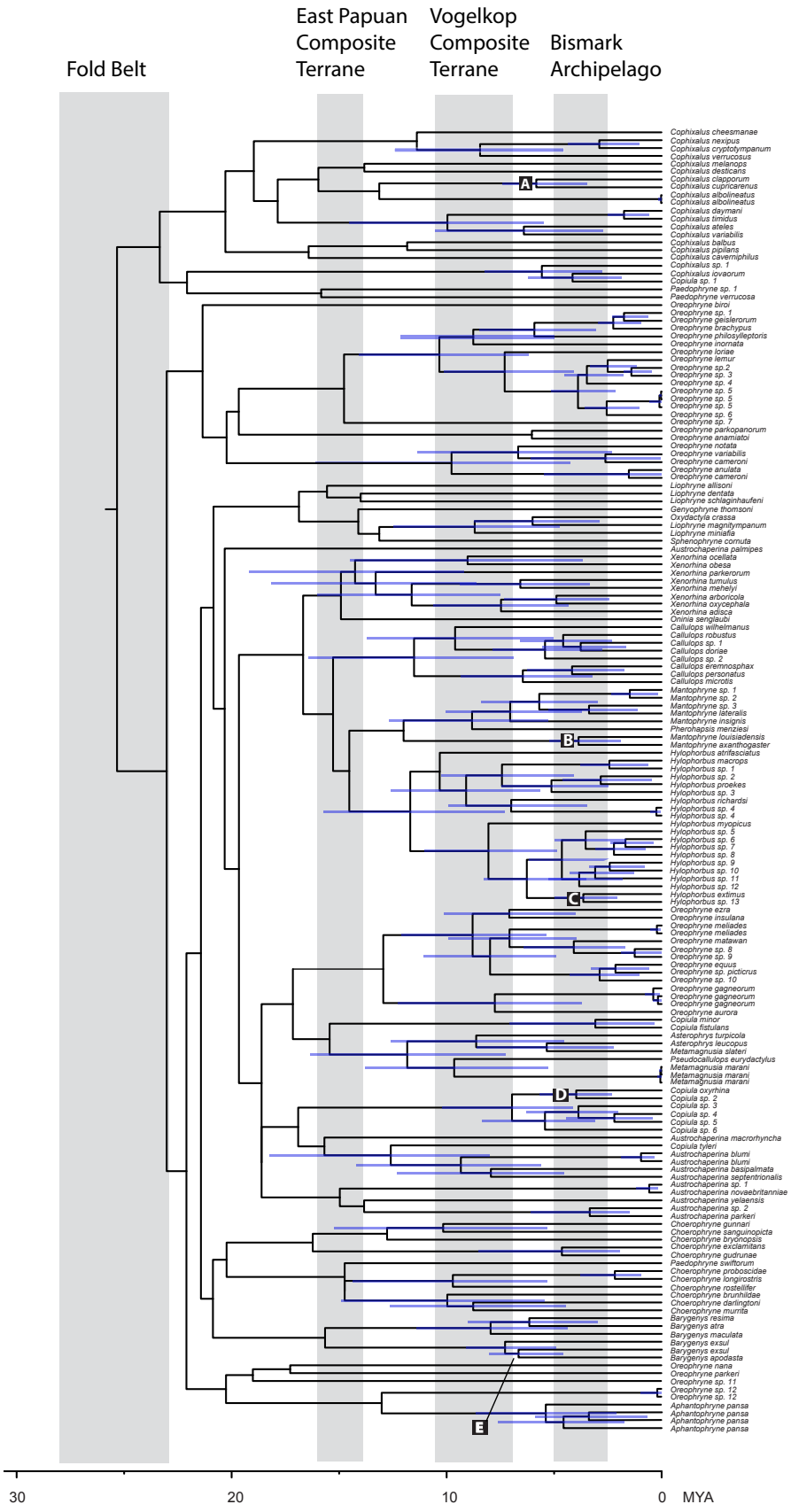

Fold Belt
East Papuan Composite Terrane
Vogelkop Composite Terrane
Bismark Archipelago
A
B
C
D
E
30
20
10
0
MYA

Figure 2.2. Time-calibrated molecular phylogeny of the Asterophryinae clade with time in millions of years before present (MYA) indicated along the bottom. Credible intervals for the age of each node are indicated by horizontal blue bars. Large confidence intervals at older nodes were left out for ease of reading; a phylogeny with all confidence intervals is provided in the supplementary materials. Letters A-E indicate the placement of calibration points used to anchor the phylogeny, which are detailed in Table 2.2. Time periods during geological events significant to the formation of New Guinea are indicated by grey background shading (from left to right: Fold Belt, East Papuan Composite Terrane, Vogelkop Composite Terrane, and Bismark Archipelago; Pigram and Davies 1987).

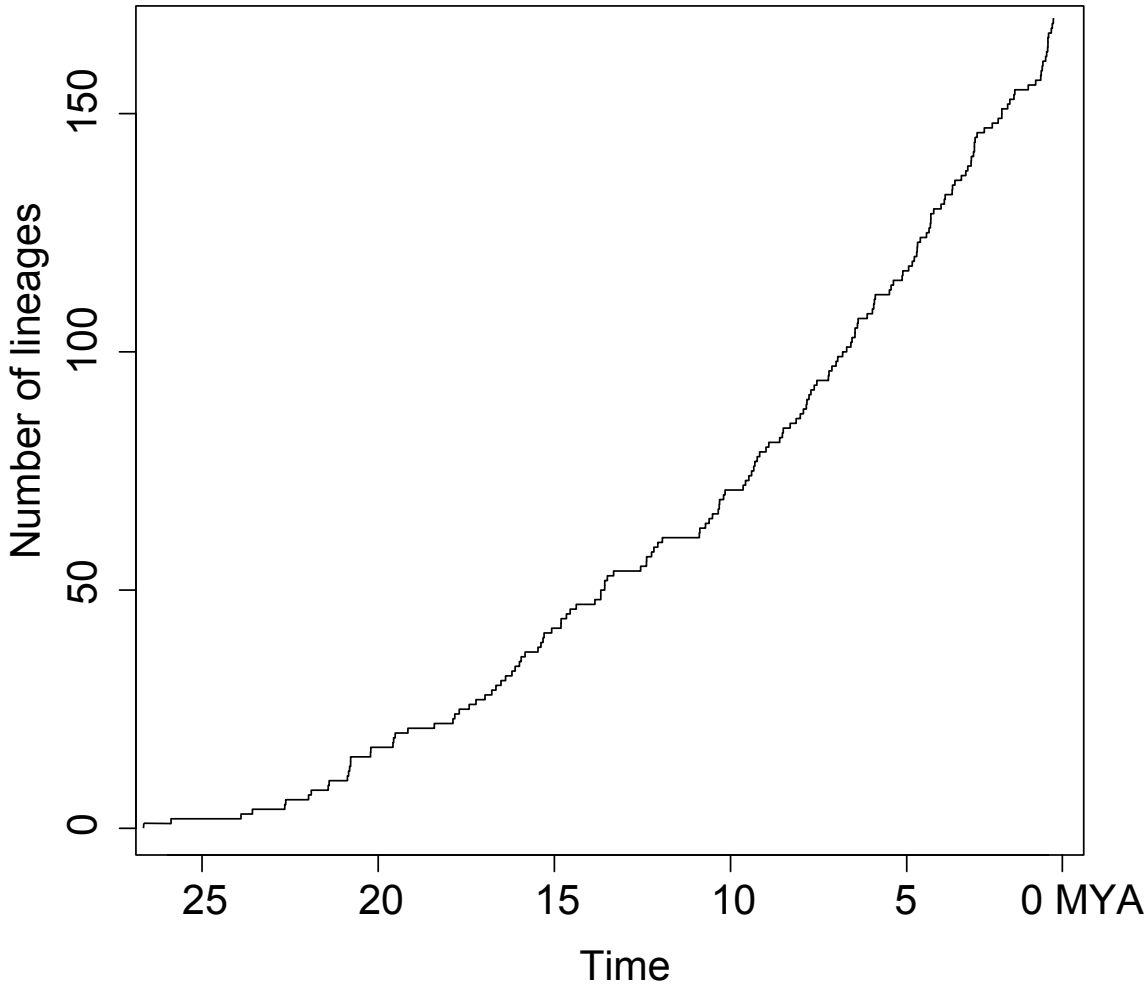


Figure 2.3. An analysis of lineage accumulation through time (LTT) for the Asterophryine phylogeny. The x-axis is time in millions of years since the inception of the clade and the y-axis indicates the number of lineages. The plot shows a concave pattern indicating that lineages accumulate faster than expected given the null hypothesis of constant rate of accumulation.

CHAPTER 3. MORPHOLOGICAL EVOLUTION AND ADAPTATION TO MICROHABITAT USE IN THE ECOLOGICALLY DIVERSE PAPUAN ASTEROPHRYINAE FROGS (ANURA: MICROHYLIDAE)

Abstract

Many animals possess specialized morphologies that allow them to better navigate their habitat and which are essential to an animal's fitness. Anurans present an interesting case as their morphology is specialized for jumping on a terrestrial environment, yet, many species utilize tree canopies, the aquatic environment, and even live underground. Here, we analyze the morphology and microhabitat correlation in a closely related, but ecologically diverse, group of frogs, the Asterophryinae. We find that species seem to be partitioning the habitat along a height gradient where subterranean species use the lowest habitats, while ground and semi-aquatic species use intermediate heights, and the shrub and tree species use the highest habitats. We also find that forelimb elements, like hand length and toe-pad width, are positively correlated with habitat so that species that live in the highest habitats have elongated forelimb elements. Furthermore, the ground species have elongated hindlimb elements and subterranean species have shortened hindlimb and forelimb elements. It also appears that many of the limb elements are evolving in response to selection for microhabitat use and is independent of phylogeny. Overall, our study highlights the importance of natural selection and convergence in explaining similarity in species traits over large temporal scales, even in morphologically specialized group like frogs.

Introduction

Animals possess specialized morphologies that allow them to better navigate their habitat. These morphologies appear to have evolved to perform particular locomotor tasks that are intimately tied a species' microhabitat use. For example, aquatic animals have flippers, flying animals have wings, and running animals have long, gracile limbs. Moreover, the need for animal's morphology to match their habitat is essential for their fitness (Arnold, 1983). The correlation between morphology and habitat use among members of a community has been studied by many (Gatz, 1979; Ricklefs et al. 1981; Miles and Ricklefs, 1984; Pianka; 1986, Voss, 1988; Losos, 1990) and has furthered our understanding of how selection is driving phenotypic evolution generating the myriad of morphologies seen today (Williams, 1972; Schluter and Grant, 1984; Grant, 1986; Losos 2009).

The most famous example of this is the Caribbean *Anolis*. The *Anolis* group has partitioned their habitat by using different substrates and have evolved varying limb morphologies that match the their microhabitat (Williams, 1972, 1983; Losos, 1990a, 1992). Similarly, the Galapagos finches have also evolved a variety of beak shapes and sizes that match their food preference (Abbott et al., 1977; Schluter and Grant, 1984; Grant, 1986; Price, 1987). The Hawaiian silversword alliance also show classic characteristics of habitat-phenotype matching where leaves have evolved a variety of structures and shapes that match a wet-dry continuum (Robichaux, 1984; Robichaux and Canfield, 1985; Carr et al., 1989). This phenomenon generating subtle aspects of morphology evolve to match ecology is called ecomorph (Schluter, 2000). Ecomorphological theory predicts that differences in morphology impose differences in performance capabilities which lead to differences in ecology or behavior (Schluter, 2000).

Table 3.1. Ecomorph categories. We designate ecomorph categories based on microhabitat use. The names refer to the microhabitat locations where frogs tend to be found. Field collectors have previously referred to these categories sometimes referring to the locomotor habits of frogs (Alt. Description in table, Zweifel and Tyler, 1982).

Microhabitat	Alt. Description	Description
Subterranean	Fossorial	Species actively dig and live underground.
Ground	Terrestrial	Species that rest and are active on land.
Semi-aquatic	Semi-aquatic	Species that actively swim in water but also use the terrestrial environment.
Shrub	Scansorial	Species that can be found on the ground but possess the ability to climb on low vegetation when active.
Tree	Arboreal	Species that rest or are active in tree canopies and tree holes.

Frogs, and amphibians in general, also seem to exhibit a tight correlation between phenotype and habitat use, in particular limb lengths (Duellman and Trueb, 1986; Stebbins and Cohen, 1995; McDiarmid and Altig, 1999; Wells, 2007; Hillman et al., 2009). Anurans present an interesting case for locomotor evolution because they are highly specialized for jumping in the terrestrial environment, yet, many species utilize habitats that impose other locomotor modes like climbing in forest canopies and swimming in the aquatic environment. Given that a majority of anurans have a conserved body plan designed for jumping what aspects of their shape, or even size, can vary to better navigate these disparate habitats?

The Papuan Asterophryinae frogs are an excellent group to study morphological evolution in response to microhabitat use. The lineage is comprised of more than 300 species and is monophyletic (Frost et al., 2006; Rivera et al., 2017). Coupled with this great species diversity the asterophryine also show ecological diversity that is atypical of a single anuran lineage. Zweifel and Tyler (1982) recognized several putative ecomorphs based on microhabitat use that include subterranean, ground, semi-aquatic, shrub, and tree forms (Table 3.1). Furthermore, it appears that many of ecomorphs have evolved independently throughout the phylogeny (Rivera et al., 2017). Here, we explore the

degree to which morphological features differ with habitat use, whether there is evidence for adaptive evolution, and assess support for the putative ecomorphs. If morphology is evolving in response to habitat use, we expect morphological traits of species belonging to the same ecomorph to be convergent. Moreover, traits relevant to locomotor performance should relate to microhabitat use. Beyond finding contemporaneous correlations between ecology and morphology, in order to support a hypothesis of adaptation, there should be evidence that these relationships are driven by selective pressures and not simply correlation resulting from relatedness.

Methods

Field Studies

Field studies were conducted during the summers of 2013 and 2014 in Papua New Guinea. Four field sites were chosen for their diverse species assemblage: Mwatebu, Milne Bay Province; Buyetai, Milne Bay Province; Maru Ruama, Central Province; and Cliffside Camp in Kamiali Village, Central Province. Fieldwork was conducted between the hours of 20:00 and 23:00 at each field site. Researchers walked slowly through the habitat listening for vocalizations made by males. Once frogs were located, the perch location was noted for later measurement and the animal caught by hand. Each animal was placed in a plastic bag with a unique ID.

Perch height (PH) measurements in centimeters were made using a tape measure, or approximated if more than 3m high. The ground was designated as zero PH, therefore frogs found in underground burrows were measured at negative PH values to indicate the depth of the burrow. Mean PH by species were used for analyses. Ecological data were collected for 29 species in (Table 3.2): Individuals were sacrificed using a 10% MS-222 solution using standard protocols and a liver sample was taken for genetic work (IACUC Protocol Number 12-1458 given to Butler at the University of Hawaii at Manoa). Animals were then injected with 10% formalin and and fixed for 24 hours. The specimens were then stored in 70% ethanol in the field and were moved to fresh 70% ethanol at the University of Hawaii at Manoa. Specimens will be deposited at the Bernice P. Bishop Museum in Honolulu, HI.

Morphological measurements on collected specimens were made by a single researcher (J. Rivera). Morphological data were collected for 28 species, the same individuals as the ecological data with the exception of *Paedophryne* sp. 3, for both sexes of microhylid frog (Table 3.2). In total, 509 individuals were measured in millimeters with a caliper for eight morphological traits that are relevant to anuran locomotor capabilities: femur length (from the end of the urostyle to the mid-knee), tibiofibula length (from the mid-knee to mid-ankle), tarsus length (from mid-ankle to proximal edge of the inner metatarsal), foot length (from the inner metatarsal to the tip of the toe), humerus length (from the articulation with the pectoral girdle to mid-elbow), radioulna length (from mid-elbow to proximal edge of the inner metacarpal), hand length (proximal edge of the inner metacarpal to the tip of the finger), 4th toe-pad width (disc width at right angle to the length of the digit), and 3rd finger-pad width and three traits that relate to habitat use: snout-vent length (SVL; from the tip of the snout to the end of the urostyle), head length (snout tip to posterior edge of the tympanic annulus), and head width (level with eardrum). Disc-pads were measured from photographs using a Zeiss Stemi DV4 Spot microscope. Digits were pressed against a glass slide with a ruler and photographed with a Canon PowerShot A650 IS mounted through the eyepiece of the microscope. Measurements were made using imageJ v1.49 (Schneider et al., 2012). Only toe-pad width was used in analysis as toe and finger pad measurements were highly correlated (linear regression; R^2=0.90)

Morphological analyses

Morphological variables were log-transformed to linearize size-corrected data and ecological data were log-transformed to reduce the skew prior to statistical analyses *per* Butler and Losos (2002). All statistical analyses were carried out in R (R Core Team 2015). Because we were interested in shape differences independent from size, we removed the size effect by subtracting log transformed morphological variables from log transformed SVL on individuals to obtain log-ratios of each morphological variable with respect to size. We used mean values by species for all morphological, size-adjusted morphology, and microhabitat use variables in analyses. To examine whether habitat use is related to morphology, we performed a multiple regression with habitat use as

independent variables and morphology or size-adjusted morphology variables as dependent variables.

We used the position of species in multidimensional morphological space to determine whether the designated ecomorphs were were more similar than species of different ecomorph designations. We did this by performing a principal component analysis (PCA) and phylogenetic PCA (Revell, 2012) using the covariance matrix of the size-adjusted data for all 28 species, and plotting PC scores. We also performed discriminant function analyses (linear and quadratic discriminant functions) to test whether the morphologies can predict ecology. We used morphological traits as the independent variable to determine how often an individual was classified under the correct ecology, the dependent variable. We obtained misclassification rates of the linear discriminant function using jackknife resampling. We dropped one observation at a time and calculated a discriminant function from the remaining data. We then classified the excluded individual using the discriminant function and tabulated whether it was correctly classified.

Phylogenetic Analyses

Because species share an evolutionary history they cannot be treated as independent data points for standard analyses, therefore, we must account for phylogeny (Felsenstein, 1985). We tested whether the evolution of morphological traits could be explained by adaptive evolution using a model-based approach (sensu Butler and King, 2004). For each morphological trait, we modeled the evolution of a continuous phenotype along the branches of a phylogeny assuming a model that accounted for stochastic changes in the phenotype, as well as an alternative that further included selection toward a hypothetical optimum value for that trait. The simplest model for stochastic evolution in a quantitative character (with no selection) can be described by a Brownian motion process where the evolutionary change in quantitative character X can be written as a stochastic differential equation:

$$dX(t) = \sigma dB(t) \qquad \text{(Equation 3.1)}$$

where *dX(t)* is the evolutionary change of a phenotypic trait (*X*) in time (*t*), the magnitude of the Brownian motion process is described by σ, and increment of random change is given as d*B*(*t*).

Alternatively, the traits may be influenced by deterministic forces such as stabilizing selection toward an optima. We use the Hansen model, which is the Ornstein-Uhlenbeck process as applied to phylogenetic comparative analysis (Equation 3.2; Martins and Hansen, 1997; Butler and King, 2004). The Hansen model has two additional parameters, one describing the strength of stabilizing selection (α), and another giving the location of the optimal trait value (Θ):

$$dX(t) = \alpha[\Theta(t) - X(t)]d(t) + \sigma dB(t) \qquad \text{(Equation 3.2)}$$

Together these parameters can be thought of as describing evolution in response to stabilizing selection toward an optimum. These models are written for each branch of the phylogeny, with different optima allowed for different branches of the phylogeny, to represent our biological hypothesis. In our analysis, the hypothesis of adaptive evolution to microhabitat type was expressed by assigning branches to optima for microhabitat use (i.e., subterranean, terrestrial, semi-aquatic, shrub, tree), according to the phenotype of the extant species, with the assignment of internal branches reconstructed assuming minimum evolution via linear parsimony (Figure 3.1).

We used the software package OUCH in the R computing environment (R Core Team, 2015) to fit our morphological data to each model to test whether our data could be best explained by an adaptive model or one of pure stochastic evolution (Butler and King, 2004; King and Butler, 2009). For each analysis, the morphological trait and a phylogeny (the pattern of relationship among species), were used as inputs in our model, with parameters for stabilizing selection, drift, and optimal trait values were fitted to the model. We compared the fit of two competing models of evolution, one of adaptive evolution to microhabitat use and the other as a stochastic process which can be described by Brownian motion. The fit of each model is assessed using Akaike Information Criteria (AIC; Akaike, 1974), AIC corrected for small sample size (AIC.c), and the Bayesian information criteria (BIC). These information criteria measure the strength of

evidence in support for each competing model (Burnham and Anderson, 2002). We assessed power via parametric bootstrap. The best-fitting model for each dataset was used to create 2000 simulated data sets used for parametric bootstrap assessment of confidence in model selection. Models were selected if they performed two or more information criteria units better than the alternative model.

We used the Asteroprhyniae ultrametric phylogeny published by Rivera et al. (2017; Figure 1) and pruned the tree to include only species for which we have morphological data using the 'ape' package in R (Paradis et al., 2004). All analyses of morphological evolution were conducted on the log-transformed and size-corrected shape variables.

Results

Ecomorphological Relationships

We find that perch height differs between ecomorphs (Figure 3.2; ANOVA: F = 141.5; d.f. = 4) where tree and shrub species use the highest perches (Figure 3.2; Tukey HSD test; P = 0.05), while semi-aquatic species use intermediate perch height (Figure 3.2; P = 2e-16), ground species use low to the ground perches (P = 2e-16), and subterranean species are found in the lowest perches, underground (Figure 3.2; P = 2e-16). The multiple regression showed that some morphologies indeed varied in relation to PH (Multiple Regression: Adjusted R^2 = 0.43; F-statistic = 30.12; DF = 419). The humerus length (P = 4.60e-6), hand length (P = 2e-16), and toe pad width (P = 2.98e-8) all had significant interactions with PH (Figure 3.3). This mirrors the results from the PCA.

The first four principal component (PC) axes explained ~90% of the total shape variation (Table 3.2). PC1 correlated positively with toe pad width, hand length, radioulna length, head length, tarsus length and tibiofibula length. PC2 correlated positively with toe pad width and contrasted with foot length, tarsus length, tibiofibula length, and femur length. PC3 correlated positively with hand and radioulna length and contrasted with head length, head width, and toe-pad width. Last, PC4 correlated positively with foot and toe-pad width and contrasted with head length and radioulna.

Figure 3.4 shows that asterophryine ecomorphs cluster in different positions of a multivariate morphological space. Subterranean species (Figure 3.4 in green) show a clear separation from the other ecomorphs along PC1.

Table 3.2. Loadings from principal components analysis of nine size-adjusted morphological characteristics for 28 species of asterophyine species*.

Size-Adjusted Variables	PC1	PC2	PC3	PC4
Femur	0.164	**-0.241**	-0.042	0.134
Tibiofibula	**0.338**	**-0.432**	0.001	0.074
Tarsus	**0.238**	**-0.352**	-0.022	-0.026
Foot	0.161	**-0.595**	0.107	**0.319**
Head length	**0.202**	-0.139	**-0.296**	**-0.855**
Head width	-0.057	-0.174	**-0.361**	0.085
Radioulna	**0.234**	-0.027	**0.260**	**-0.253**
Hand	**0.277**	0.120	**0.777**	-0.140
Toe-pad width	**0.755**	**0.460**	**-0.312**	**0.263**
Percent variance explained	39.41	28.26	11.88	10.16

*Note: Substantial loadings are marked in bold.

The results of a multivariate Discriminant Function analysis demonstrates that the average percent of individuals from the samples that were classified correctly was 89%. The jackknife cross-validation method classified the samples correctly 87% while the quadratic discriminant analysis model containing scores for all morphological traits performed best with 90% of the species classified correctly. The majority of the misclassification came from classifying shrub species into the ground category and, to a lesser extent, misclassifying shrub species into the tree category.

Plots of PCA scores indicate that subterranean, ground, and tree ecomorphs are distinct from each other, the shrub ecomorph overlaps in morphospace with tree and ground ecomorphs (Figure 3.4). This is also supported by the misclassification of the shrub ecomorph into the ground ecomorph in the discriminant function analysis. Furthermore,

the shrub ecomorph seems to be split into two distinct clusters, the *Cophixalus* genus, filled teal polygon, and the *Choerophryne* genus, the filled blue polygon. These two shrub clusters do not differ in head length (Welch Two sample t-test: t=1.35; df=64.38; *P* = 0.18) and toe pad width (Welch Two sample t-test: t=-1.68; df=60.34; *P* = 0.10) nor do they differ in perch height (Welch Two sample t-test: t=-0.009; df=54.81; *P* = 0.99). However, these two shrub groups differ significantly in all other morphological traits. This is supported by a Welch Two Sample t-test with a Bonferroni correction for all morphological traits (femur: *P* = 5.75e-9; tibiofibula: *P* = 2.2e-16; tarsus: *P* = 1.4e-9; foot: *P* = 2.3e-16; head width: *P* = 2.03e-3; radioulna: *P* = 8.2e-5; hand: *P* = 1.5e-4).

Evolutionary Analyses for Morphological Traits

The evolutionary analyses confirm that some aspects of morphological traits of the asterophryine are best explained by a model of divergent selection between the microhabitat types. The adaptive model, with microhabitat as the selective factor, was the best-fitting and strongly supported model for the femur, tibiofibula, radioulna, and toe pad width (Table 3.3). The tarsus showed a similar pattern in that the adaptive model best fit the data, but the support was not as strong (Table 3.3). In contrast, we found no evidence of adaptive evolution for foot length, head length, head width, and hand length. For these traits, the BM was the best-fitting and strongly supported model.

For the traits in which the microhabitat model was best, the predicted optima indicate the evolution of morphological differences among ecomorphs. Semi-aquatic species are evolving towards large optima while subterranean and tree species are evolving towards a small optima for femur length (Table 3.3).

The shrub and ground species are evolving towards long optima for radioulna while subterranean and semi-aquatic species are evolving towards smaller optima for radioulna (Table 5S).

Lastly, the tree, shrub, and semi-aquatic species are evolving towards large optima for toe-pad width while subterranean and ground are evolving towards small toe-pad width optima (Table 5S).

Table 3.3. Model fit statistics for morphometrics. The model with the best fit, using AIC.c, is listed as 0, with ΔAIC.c values listed for all other models. Bootstrap model selection frequencies based on 2000 bootstrap replicates are included in parentheses.

Variables	Microhabitat	BM
Femur	0 (75)	4.1 (25)
Tibiofibula	0 (75)	3.3 (25)
Tarsus	0 (73)	0.9 (27)
Foot	10 (75)	0 (25)
Head length	8.8 (66)	0 (34)
Head width	7.7 (76)	0 (24)
Radioulna	0 (84)	21.6 (16)
Hand	4.8 (77)	0 (23)
Toe-pad	0 (64)	11.8 (36)

Discussion

We have demonstrated ecological differences between the categories of frogs described by Zweifel and Tyler (1982). These ecological differences translate into morphological differences, and moreover we find support for adaptive evolution driving evolution of these traits. Therefore, we have clearly strong support for the existence of "ecomorphs" among the Papuan microhylid frogs.

Relationship between Morphology and Microhabitat Use

A fundamental prediction of ecomorphological studies is that morphology will evolve to match ecology when specialization provides a fitness advantage (Arnold, 1986; Wainwright, 1994). Indeed, we have found that enlarged toe pads are strongly associated with those species that can climb, tree and shrub species, as well as the semi-aquatic species. It has been shown by others that an enlarged toe pad area can provide enough

sticking force so that some species of frogs can hang upside down (Emerson and Diehl, 1980). This would be beneficial for those species that possess an arboreal lifestyle as they tended to call from perches greater than three meters. Enlarged toe pads were also correlated with the semi-aquatic species *Austrochaperina palmipes*. This species was usually found clinging to slippery rock walls in fast moving streams. Emerson and Diehl (1980) showed that frogs with enlarged toe pads can cling to rough substrates, like bark, by interlocking to stick to the surface. This may be the case for *A. palmipes* as they were usually found firmly pressed against a rock.

The tibiofibula seems to be closely related to ecology. The tibiofibula is the out-lever of the lower leg extensor muscle in anurans. Emerson (1976) found that burrowing frogs have the shortest tibiofibula when compared to frogs that possess other lifestyles. We found this to be the case and also found that ground species and the shrub *Cophixalus* species all had an elongated tibiofibula when corrected to size. The elongated tibiofibula of the ground and some shrub species provides a greater distance advantage allowing the limb to move at greater speeds. This would be advantageous if jumping is the primary form of locomotion for an animal (Emerson, 1988). Conversely, the short tibiofibula seen in subterranean species provides a greater force advantage. This would be especially useful if the subterranean species use their hindlimbs for displacing soil and burrowing (Emerson, 1976). In fact, it is the case that all ground species have elongated hindlimb elements while subterranean species have shortened hindlimb elements.

The radioulna forelimb element also differed between ecologies. The ground and the shrub species had the longest radioulna while the subterranean species had the shortest. Emerson (1988) also found this to be the case where hopping frogs had a longer radioulna while the burrowers had the shortest. Emerson (1988) showed that species that walk have longer radioulna, and forelimbs in general, than those that burrow or hop.

Evolutionary Convergence

What is most interesting is that many of the ecomorphs have evolved multiple times yet share a similar design. This is clearly a result of independent, convergent evolution driven by selection for microhabitat use and supported by the evolutionary

analyses. For example, ground species have large optima for hindlimb elements that correlate to their terrestrial lifestyle. Likewise, subterranean species, which have evolved four independent times, have small optima for hindlimb and forelimb elements that are common in fossorial animals to aid in digging. Toe-pad size and radioulna length are also driven by microhabitat use as arboreal species, like the tree and shrub ecomorphs, have share large optima for digital discs and radioulna length
despite these ecomorphs arising independently five times.

The shrub ecomorph has evolved four independent times in our phylogeny (Figure 3.1) but is not as distinct morphologically as the other ecomorphs (Figure 4). The shrub ecomorph overlap in morphospace with ground and tree ecomorphs. This is not surprising as the shrub ecomorph uses both the terrestrial and arboreal environment. Therefore, it is reasonable to expect shrub species to share some aspects of morphology in common with both tree and ground species. We indeed find that this is the case. For example, shrub species possess enlarged toe pads, like tree species, but also elongated femurs like ground species. We see a separation within the shrub ecomorph that is associated with relatedness. The shrub species in the teal polygon (Figure 3.4) are all part of the *Cophixalus* genus while the species in the blue polygon is part of the *Choerophryne* genus. Furthermore, they differ in all other morphologies, aside from head length and toe pad width, where the shrub *Cophixalus* have elongated hindlimb elements and the shrub *Choerophryne* have elongated forelimb elements. Despite possessing different morphologies, these two subsets of the shrub species do not differ in any ecological parameter measured. This separation may reflect ancestral states as the *Choerophryne* genus is solely a shrub specialist while the *Cophixalus* genus is predominantly the ground ecomorph with the shrub ecomorph evolving throughout the clade. This separation also emphasizes the importance of particular traits to a species' lifestyle. These two groups of shrub species use high perches, and therefore need to climb, emphasizing the large toe-pads. It is also the case that these shrub species are rarely found on the ground and jumping may be less crucial as a locomotor tasks, which is why the same convergence of morphology is not found in the hindlimbs.

Conclusion

Here, we have shown that the asterophryine ecomorphs are distinguishable in size-independent morphospace. Furthermore, it appears that these ecomorphs have evolved repeatedly several times throughout the asterophryine lineage and morphological traits converge on microhabitat use. Coupled with the evolutionary modeling, these results suggest that ecological diversification is contributing to the evolution of biodiversity of this clade.

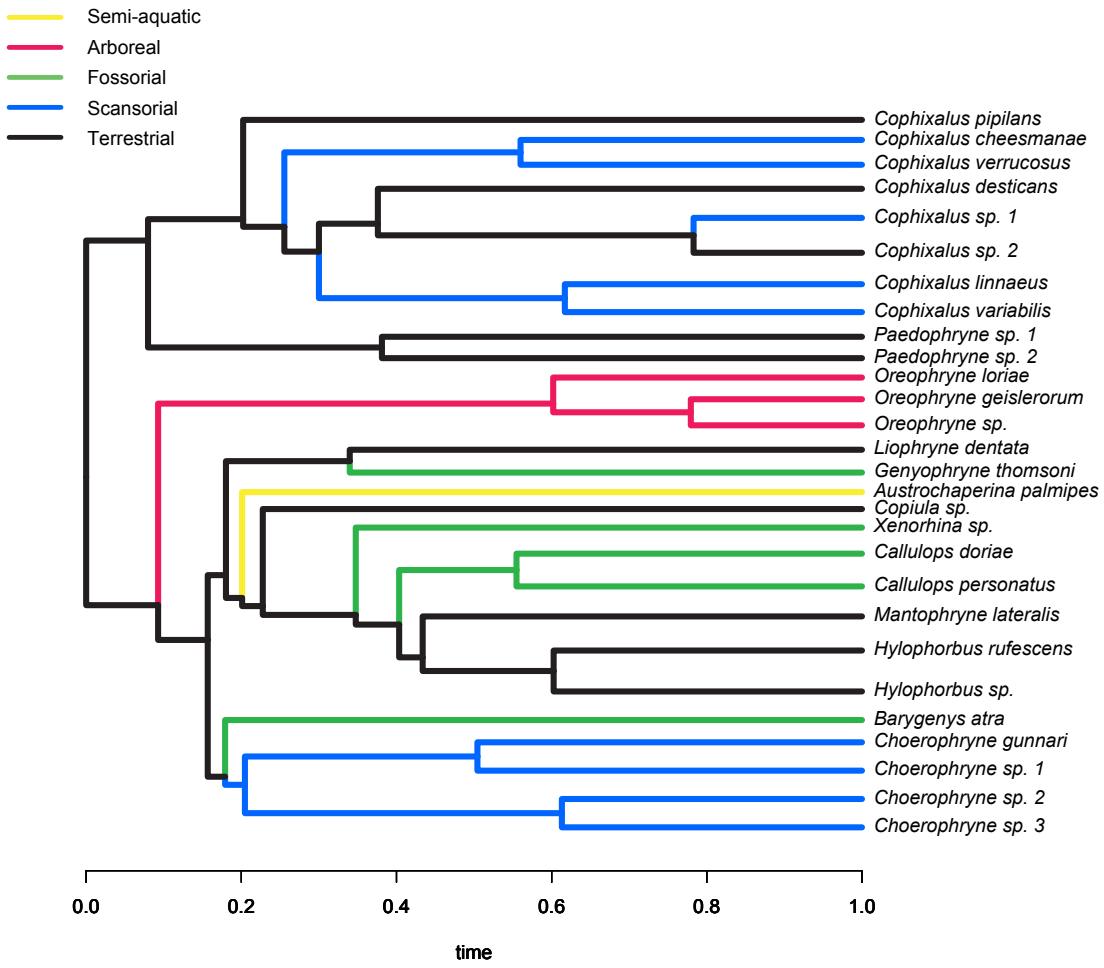


Figure 3.1. The *Asterophyinae* phylogeny used in this study with branch lengths proportional to time (modified from Rivera et al., 2017). Microhabitats are mapped onto the phylogeny represented by different colors.

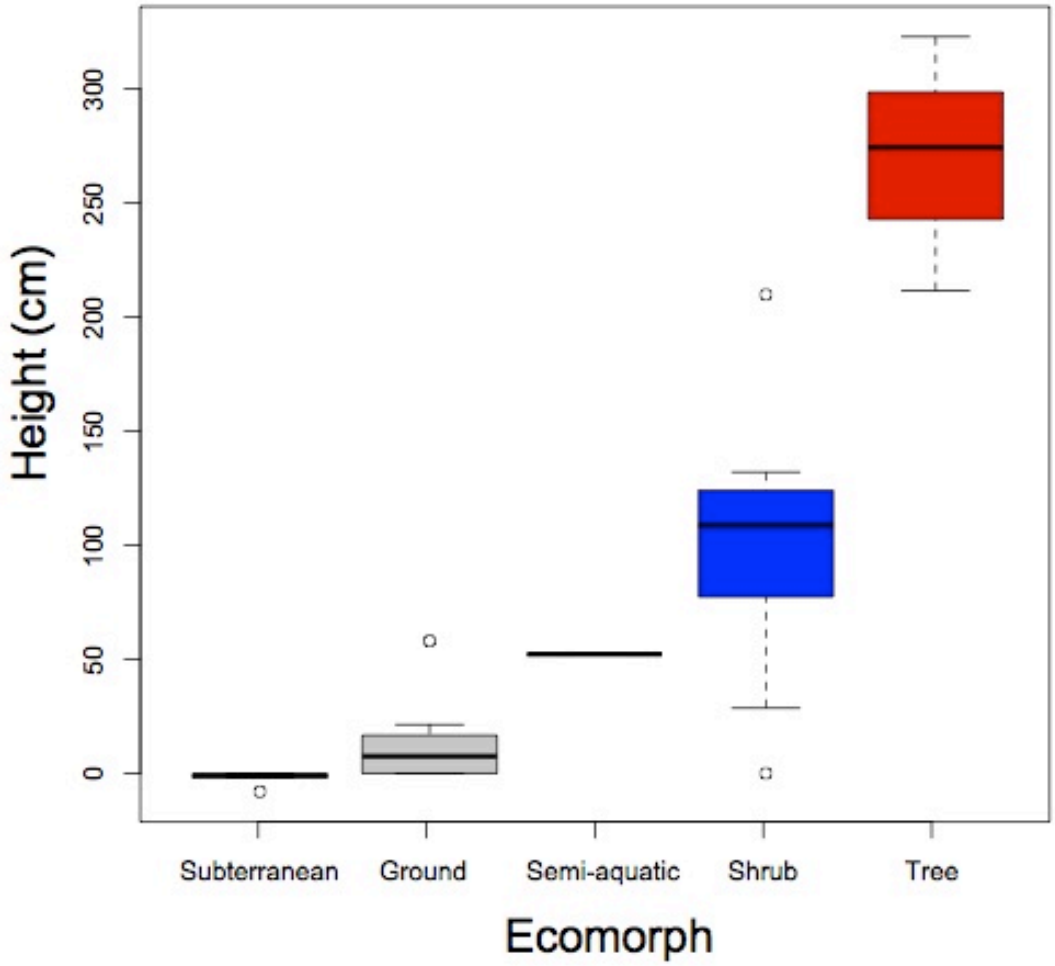


Figure 3.2. Perch height of species means grouped by ecomorph where red = tree, blue = shrub, and gray = ground.

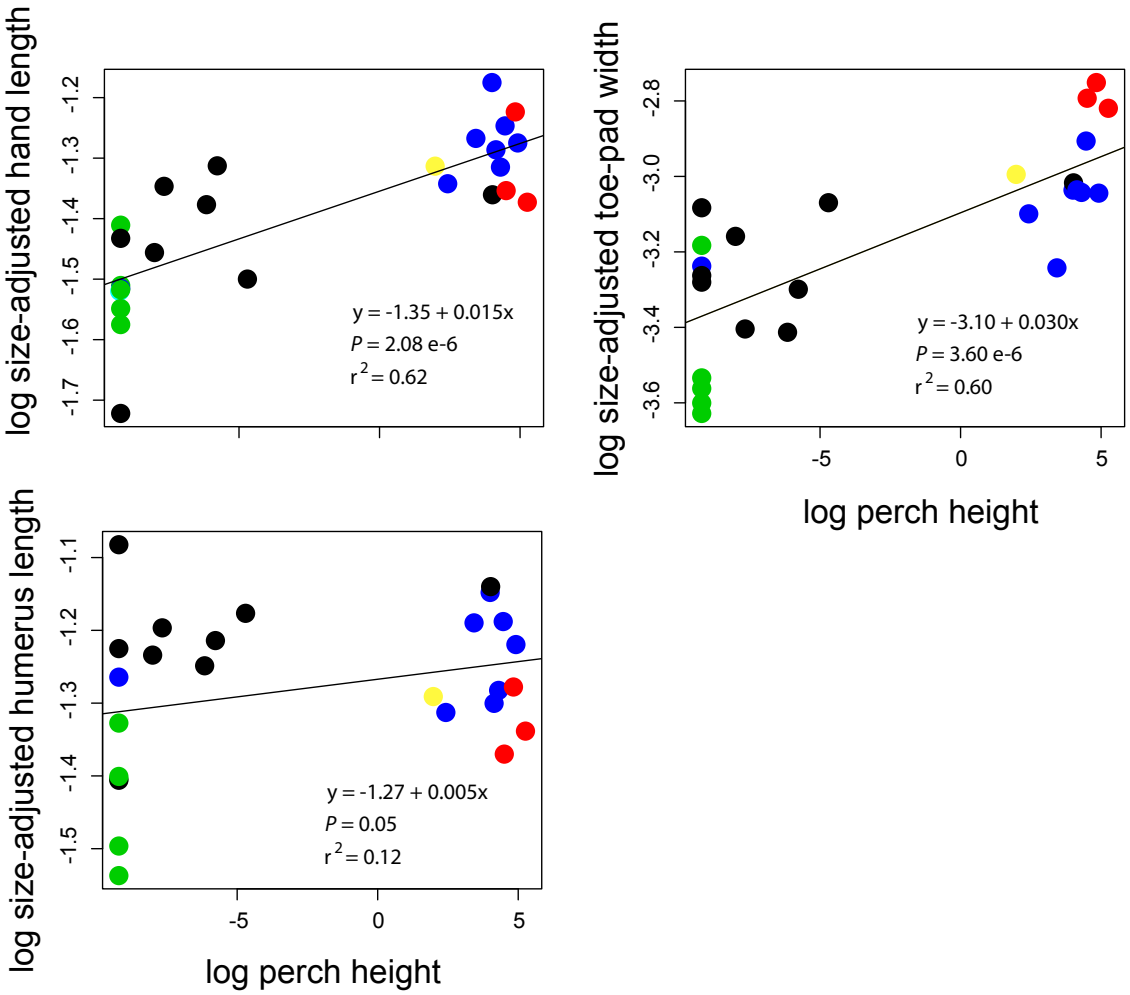
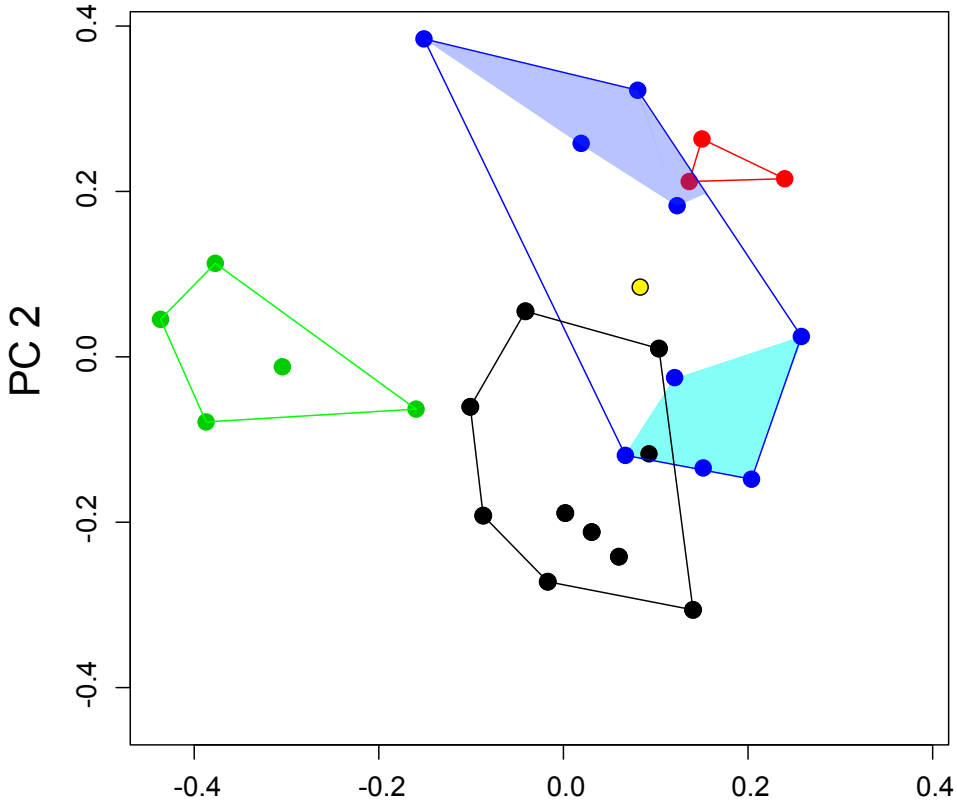


Figure 3.3. Regression of interspecific variation in size-adjusted traits as a function of perch height colored by ecomorph where green = subterranean, blue = shrub, yellow = semi-aquatic, black = ground, and red = tree. The linear model, p-value, and R-squared are shown in the figure.


PC 2
0.4
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
0.4

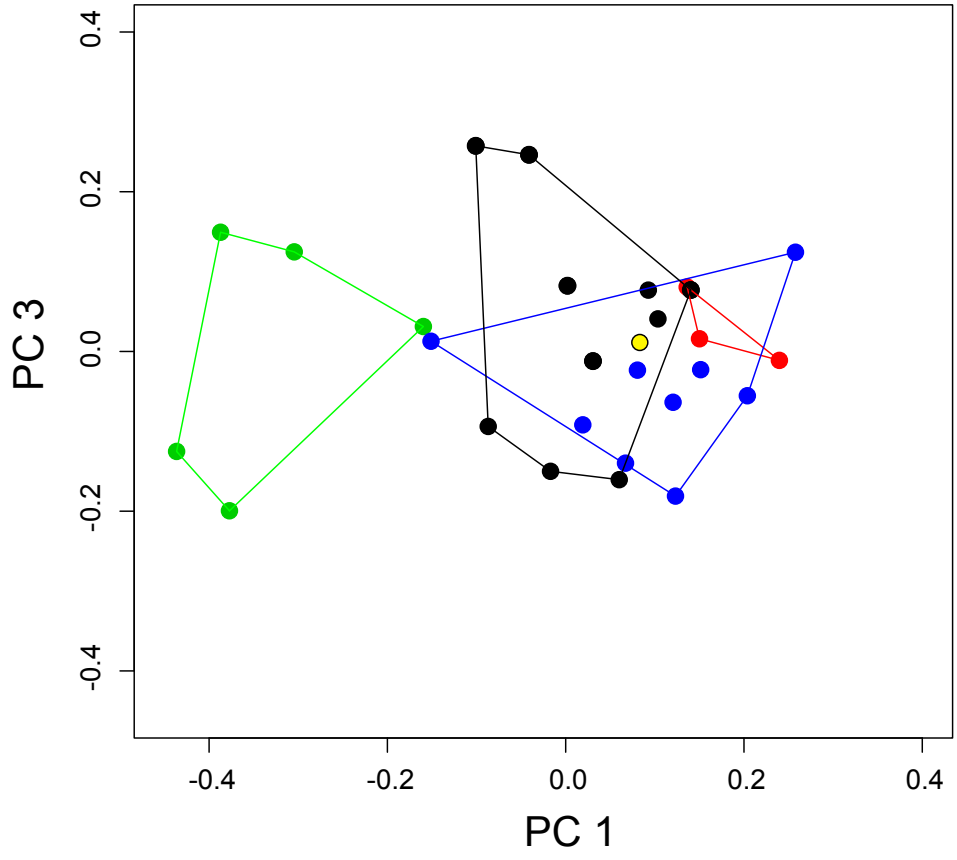

PC 3
0.4
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
0.4
PC 1

Figure 3.4. Plots of principal component scores for PC1 vs PC2 (top) and PC1 vs PC3 (bottom) colored by ecomorph where green = subterranean, blue = shrub, yellow fill = semi-aquatic, black = ground, and red = tree. These color codes are used throughout the manuscript. The filled teal polygon represents the *Cophixalus* genus while the filled blue polygon represent the *Choerophryne* genus.

CHPATER 4. JUMPING, SWIMMING, AND CLINGING PERFORANCE VARIATION ACROSS ECOMORPHS IN ASTEROPHRYINAE FROGS FROM PAPUA NEW GUINEA

Abstract

Locomotor performance is essential to the fitness of many animals and tight correlations exist between performance and morphology. However, species may also be required to perform other tasks, like climbing and swimming, in addition to their primary form of locomotion. Here, we analyze three performance tasks, jumping, swimming, and clinging, in a closely related group of frogs that utilize disparate habitats, the asterophryine. We find that the shrub and tree ecomorphs cling at larger angles than other ecomorphs. The shrub and tree ecomorph also possess elongated forelimbs and enlarged toe-pads that aid in their arboreal lifestyle and were also found to be some of the smallest species. The semi-aquatic ecomorph excelled at swimming and also possess elongated hands, enlarged toe-pads, and interdigital webbing that allow them to propel themselves through the water more readily. The ground ecomorph were able to jump that farthest distances which was accomplished by elongating the hindlimbs. Our study demonstrates that performance differences and trade-offs exist between ecomorphs and that particular morphological traits play a critical role in achieving these locomotor tasks. Last, swimming and jumping performance are driven by selection to microhabitat use.

Introduction

Locomotor performance is essential to the fitness of many animals (Arnold, 1983). In order to catch dinner, escape predators, or compete for mates, animals jump, swim, climb, or sprint. Given the diversity of locomotor performances found in animals one may expect a variety of selective pressures to shape performance capabilities. For example, predator avoidance seems to have shaped the high speeds seen in the c-start of fishes (Langerhans, 2009a), swimming speed in insects (Strobbe et al. 2009), and sprint speed in lizards (Miles, 2004, Scales and Butler 2016). Foraging mode also influences performance capabilities. Ambush predators are quick but move infrequently while pursuit predators have have high stamina but may be slow (Miles et al., 2007, Scales and Butler 2016).

Animals achieve these tasks by evolving specialized morphologies that allow them to excel at a particular locomotor tasks (Gatz, 1979; Ricklefs et al., 1981; Miles and Ricklefs, 1984; Pianka, 1986; Voss, 1988; Losos, 1990; Wainwright and Reilly, 1994). Broadly, animals capable of flying have wings, digging animals have short powerful limbs, and swimming animals have flippers. Selection also fine-tunes morphology to match locomotor demand at smaller scales. For example, anolis lizards that possess long limbs have higher sprint speeds than those species with short limbs (Losos, 1990a). Indeed, locomotor capabilities are intimately tied to morphology and driven by selection.

Anurans present an interesting case because they possess highly specialized morphologies, most notably the derived elongated pelvis and elongated hind limbs, uniquely optimized for jumping. However, this may conflict with other forms of locomotion as many anurans can also swim, dig, climb, and glide (Rand, 1952; Zug, 1972; 1978; Emerson, 1979; 1978). Studies on clinging and jumping by Emerson (1991) found no differences in locomotor capabilities nor did she find trade-offs across and within closely related species that are specialized to live in trees. Similarly, Gomes et al. (2009) found little difference in jumping performance within clades, however, little ecological diversity existed in the clades measured. What is largely missing from the literature is to determine if locomotor performance differs between species that utilize disparate habitats but are closely related. Furthermore, we do not know if species are

able to maximize all performance capabilities or do trade-offs exist so that only a single performance task can be maximized at the expense another. Last, the majority of the literature has focused on hindlimb morphology to understand how morphology relates to performance, but little work has been done in understanding how postcranial morphology relates to performance.

The Papuan Asterophryinae frogs present an excellent example to study the further diversification of forms within a closely related group of anurans. The asterophryine lineage is monophyletic and ecologically diverse, atypical of a single anuran lineage (Rivera et al., 2017). Ancestrally direct-developing, the clade has diversified into over 300 species that use a variety of microhabitats: trees, shrubs, streams (semi-aquatic), the ground, and subterranean burrows (Chapter 2*)*. Moreover, these frogs have evolved morphologies that correspond to their preferred microhabitats or "ecomorphs." Tree and shrub species have elongated forelimbs and enlarged toe-pads, semi-aquatic species have elongated hands and interdigital webbing, ground species have elongated hindlimb elements, and subterranean species have shortened hind limbs (Chapter 2*)*. While these morphological differences are quite suggestive, we do not know how these morphologies relate to performance capabilities nor do we understand their ecological relevance. Here, we conduct performance tests to determine if locomotor capabilities differ between ecomorphs to provide performance advantages relative to their lifestyle. We discuss these differences in relation to biomechanical models and explore the role of adaptive evolution in producing the diversity of performance differences observed in this clade.

Methods

Fieldwork and Morphometrics

Field studies were conducted during the summers of 2013 and 2014 in Papua New Guinea. Four field sites were chosen for their diverse species assemblage: Mwatebu, Milne Bay Province, Buyetai, Milne Bay Province, Maru Ruama, Central Province, and Cliffside Camp in Kamiali Village, Central Province. Fieldwork was conducted between the hours of 20:00 and 23:00 at each field site. The researcher walked slowly through the

habitat listening for vocalizations made by males. Frogs were collected by hand, placed in plastic bags and transported to the laboratory between one and four days after evening searches. Upon arrival, frogs were individually housed within plastic containers with air holes and were kept moist. Each container was housed in the laboratory and kept at the average temperature of the site of collection. The sex of all individuals were verified through inspection of vocal slits. The temperature in the field varied between 20.9-26.1 °C and experiments were conducted between 22.1-27.3 °C. The temperature range that the experiments were conducted in are well within the range of peak performance for tropical frogs (Navas et al., 2008). After performance tests were conducted, individuals were sacrificed using MS-222 using standard protocols (IACUC Protocol Number 12-1458 given to Butler at the University of Hawaii at Manoa). Animals were injected with 10% formalin and were fixed for 24 hours. Specimens were then stored in 70% ethanol in the field and moved to fresh 70% ethanol at the University of Hawaii at Manoa. Specimens will be deposited at the Bernice P. Bishop Museum in Honolulu, HI.

Morphological measurements on specimens were made by a single researcher (J. Rivera). Morphological data were collected for 24 species for both sexes of microhylid frog (Table 3S). In total, 509 individuals were measured in millimeters with a caliper for 11 morphological traits: snout-vent length (SVL; from the tip of the snout to the end of the urostyle), femur length (from the end of the urostyle to the mid-knee), tibiofibula length (from the mid-knee to mid-ankle), tarsus length (from mid-ankle to proximal edge of the inner metatarsal), foot length (from the inner metatarsal to the tip of the toe), head length (snout tip to posterior edge of the tympanic annulus), head width (level with eardrum), humerus length (from the articulation with the pectoral girdle to mid-elbow), radioulna length (from mid-elbow to proximal edge of the inner metacarpal), hand length (proximal edge of the inner metacarpal to the tip of the finger), 4th toe-pad width (disc width at right angle to the length of the digit), and 3rd finger-pad width. Disc-pads were measured using a Zeiss Stemi DV4 Spot microscope. Digits were pressed against a glass slide and a ruler was placed on the slide. We mounted a Canon PowerShot A650 IS onto the microscope to photograph the disc-pads. Pictures were then measures on imageJ v1.49 (Schneider et al., 2012). Only toe-pad width was used in analysis as toe and finger

pad measurements were highly correlated (linear regression; R^2=0.90). For measurement details see Chapter 3.

From the 11 morphological traits measured only the most relevant traits to habitat use were chosen (see Chapter 3). These include: foot length, tarsus length, tibiofibula length, hand length, and toe-pad width. We performed a multiple regression using morphology as the independent variables and performance as the dependent variable. We also added the interaction between morphology and ecomorph to determine if ecomorph categories contained any explanatory power. We also performed a Canonical Correlation Analysis to identify and measure the association among the morphological and performance variables.

Biomechanical Models

By using biomechanical models we are able to make *a priori* predictions about the relationship between morphology and performance which should hold, by extension, to morphology and ecology (Wainwright, 1987; 1988).

Distance jumped (λ) by a frog can be described by the formula for ballistic motion, which describes distance as a function of velocity (v) at takeoff, takeoff angle (α), and gravity (g):

$$\lambda = v^2 \sin(2\alpha) / g \qquad \text{(Equation 4.1)}$$

Given that gravity is a constant, frogs must vary in either initial velocity, or takeoff angle, or both to achieve differences in total jump distance. Furthermore, we expect elongated hind limbs in species that are able to jump farther to accommodate longer tendons. It is known that anurans use tendons to store energy prior the limb extension phase of the jump (Astley and Roberts, 2012). Therefore, longer tendons may hold more elastic energy that can be transferred to achieve a longer jump while shorter limbs, and tendons, may produce less energy for the jump.

In frogs, clinging capabilities are a function of Stefan adhesion which describes the stress generated when two parallel discs with fluid between them is attempted to be separated (Bikerman, 1971). Stefan adhesion (f) is described as:

$$f = 0.75\mu\alpha^2 / h^2 t + 2V/h \qquad \text{(Equation 4.2)}$$

Where μ = the viscosity of the liquid, α = the radius of the disc, t = seconds until separation, h = the initial distance between the discs, and V = the surface tension (Bikerman, 1971). We expect the values for viscosity (μ), distance between discs (h), and surface tension (V) to be in common between species (Emerson and Diehl, 1980; Federle et al., 2006) and assumed no difference in time (t), therefore, radius of disc (α) is the only components that will vary. We expect to find species that are able to cling at large angles have enlarged toe-pads and can cling for longer periods of time than those that lack digital discs.

In comparing swimming performance, thrust (T) is arguably the most prominent force in aquatic locomotion. It is generated by pushing against the surrounding medium, in our case water, and described as:

$$T = mv/t \qquad \text{(Equation 4.3)}$$

Where m = mass, v = velocity, and t = time. We expect that species with large thrust values will have aquatic adaptations that include paddle like appendages and streamlined bodies. Furthermore, we expect species to have elongated hind limbs which will allow for more powerful kicks.

Performance Data

Overview: For each individual we collected data on performance in jumping, swimming, and clinging. These behavior were chosen because they are likely to be divergent across species using different microhabitats. Jumping is important for almost all frog species (Gans et al., 1966; Zug, 1978; Emerson, 1979) but trade-offs may exist if species utilize other forms of locomotion as well.

Jumping: Each individual frog underwent 3-4 jump session, between a day and four days after collection. In each session the frog underwent two trials, then were allowed to rest between half a day to a full day and jumped again. Frogs were tested in

the morning (0900-1200h) and afternoon (1300-1700h). Individuals were randomized within jump session and only a single jump that represented maximum effort of each individual was used for data analysis.

The complete takeoff phase of each jump was recorded on two, one dorsal and one lateral, IDT Vision N4 high-speed camera at 250 frames per second. This frame rate is generally appropriate for small jumping vertebrates (Kuo et al., 2011). We used the Motion Studio x64 software from IDT Vision to record high speed jumps. The total jump length was captured on a Sony HD Handycam camcorder and longest jump for each individual's trial was used as the "best" jump. We recorded the force produced by each jump using a force plate (Kistler type 9865B, Kistler AG, Winterthur, Switzerland) for the summer of 2014. We used the BioWare Software (Kistler type 2815A, Kistler AG, Winterthur, Switzerland) to record the force produced.

Clinging: We designed a cling apparatus by gluing a metal hinge to the bottom of a Teflon®-coated non-stick frying pan (28.5 cm diameter, 6 cm deep). A Teflon®-coated pan was used because it has a similar coefficient of friction as waxy leaves that are common in rainforests (Emerson, 1991). Frogs were placed at the center of the leveled pan (0°) then inverted at a constant rate up to 180°. The angle of the pan was noted at the moment in which each individual exhibited a characteristic behavior which we called "crouching." The frog increased contact area between its ventral surface and the pan, engaging surface area beyond just the digital pads, which we took to indicate the loss of traction. Each frog was tested 3 times to ensure accurate estimates of performance but the largest angle was taken for analyses. We used circular statistics to analyze the angular data.

Swimming: Each individual frog underwent 3 swim sessions the following day after jumping and clinging. Burst swim performance was elicited by releasing frogs at one end of a aquarium (50 cm long by 30 cm wide by 25 cm tall) filled with water to a depth of 15 cm. Swim performance was captured from above using a Sony HD Handycam camcorder at 30 frames per second, due to the slower velocities associated with swimming. Only the *Paedophryne* genus was not able to swim at the surface therefore their swim measurements are from the bottom of the tank as opposed to the surface of the water, like in all other genera.

Data Extraction from Videos and Performance Variable

We analyzed the 3D high speed videos using the ProAnalyst motion studio software (Xcitex Inc., Woburn, Massachusetts, USA) to obtain velocity, acceleration, and takeoff angle. Velocity and acceleration were obtained by finding the resultant vector from the x, y, and z direction vectors for the best jump from each individual from the high speed videos.. We examined the following variables for jumping (i) takeoff angle, (ii) takeoff velocity, (iii) acceleration at takeoff, and (iv) force produced at jump. We used ImageJ 1.8.0 (Schneider, 2012) to digitize the swimming videos. In swimming, we collected data on (i) velocity and (ii) stroke time and finally for clinging our sole performance variable was maximum clinging angle.

Evolutionary Analysis

Species cannot be treated as independent points due to their shared evolutionary history (Felsenstein, 1985), therefore, we used comparative methods to account for shared history. We compared the fit of Brownian motion and Hansen models assuming the Rivera et al. (2017) phylogeny to determine which evolutionary model best explained the data. Brownian motion described evolution as a purely stochastic process where the Hansen models are Ornstein-Uhlenbeck processes that model evolution under stabilizing selection (see chapter 3 for details; Martins and Hansen, 1997; Butler and King, 2004).

We used the Asteroprhyniae phylogeny published by (Rivera et al., 2017, Figure 1) and pruned the tree to include only species for which we have performance data using the 'ape' package in R (Paradis et al., 2004). We constructed two hypotheses for the evolution of morphological traits. The first was an adaptive model where traits are evolving in response to microhabitat use. The microhabitat model contained 5 optima based on microhabitat use of each species: subterranean, ground, semi-aquatic, shrub, and tree which place different selective pressures on the species associated with them (Figure 1). The second model was Brownian motion which explains phenotypic evolution by drift therefore, there are no assumptions were made about adaptation.

Each evolutionary model was fit to the data and phylogeny using the package OUCH (Butler and King, 2004; King and Butler, 2009) in the R computing environment

(R Core Team, 2015). We assessed the fit of each model using Akaike Information Criteria (AIC; Akaike, 1974), AIC corrected for small sample size (AIC.c), and the Bayesian information criteria (BIC). These information criteria measure the strength of evidence in support for each competing model (Burnham and Anderson, 2002). We assessed power via parametric bootstrap. The best-fitting model for each dataset was used to create 2000 simulated data sets used for parametric bootstrap assessment of confidence in model selection. Models were selected if they performed two or more information criteria units better than the alternative model.

Results

Performance

Jumping: Absolute jump distance did not vary significantly between ecomorphs with the exception of ground species jumping absolutly further, ~37cm, than subterranean species who jumped ~11.5 cm (Figure 2; ANOVA with *Post Hoc* Tukey test HSD: $F = 5594$; Df = 4; $P = 0.003$). We found no difference in absolute jump distance between the shrub (22.37 cm), semi-aquatic (23.96 cm), and tree species (28.15 cm). However, we found that ecomorphs varied substantially in jump distance relative to their size (Figure 2; Table 4S). Ground species, jump the most body lengths, ~15 body lengths on average with some jumping as much as 25 body lengths, while subterranean species jumped the short distances (~4 body lengths) relative to their size. Tree and shrub species both jump ~12 body lengths and semi-aquatic species jumped ~7 body lengths.

We found that some of the morphological characters explained variation in size corrected jump distance. An ANOVA shows that species with elongated tibiofibulae and enlarged toe-pads are able to jump further distances, when corrected for size, than those with short tibiofibulae and small toe-pads (Table 9S, 10S).

Ballistic motion is governed by initial velocity and take-off angle. Species were for the most part highly conserved in take off velocity. All ecomorphs performed with the same take off velocity of 1.82 m/s with the exception subterranean species. Species that belong to the subterranean ecology jumped at a slower velocity, 1.2 m/s, compared to all

other ecologies (ANOVA with *Post Hoc* Tukey HSD: $F = 8.03$; Df = 4; $P = 3.4e\text{-}6$). This difference is driven by a single species, *Callulops doriae* (Table 4S).

Ground species jumped at a larger take-off angle than all other ecomorphs, ~40°, whereas the jump angles of other ecomorphs were ~31° (Figure 2; Circular Analysis of Variance: $F = 6.39$; Df = 4; $P = 5.50e\text{-}5$).

Similarly, we found no differences between acceleration and ecomorphs, with the exception of *C. doriae* (ANOVA with *Post Hoc* Tukey HSD: $F = 303.24$; Df = 4; $P = 1.69e\text{-}8$).

Ground species jumped at a larger angle, ~40°, when compared to other ecologies, ~31° (Figure 2; Circular Analysis of Variance: $F = 6.39$; Df = 4; $P = 5.50e\text{-}5$).

Last, we found that tree and shrub species produced the smallest forces when corrected for size while the semi-aquatic species produced the largest forces. Terrestrial and subterannean species produced intermediate forces (ANOVA with *Post Hoc* Tukey HSD: $F = 14.96$; Df = 4; $P = 2.44e\text{-}11$).

Clinging: We found a correlation between microhabitat use and clinging ability. The species that are able to climb, tree and shrub species, were able to cling at larger angles while the subterranean species performed the poorest at clinging (Figure 4.3,Table 7S).

There also appears to be relationships with some of the morphologies and clinging ability. Toe-pad width had a positive relationship with cling performance (Table 11S) while foot length had a negative relationship with cling performance (Table 12S). We also found that overall size of a species influences clinging ability and interacts with microhabitat use. Smaller species can cling at steeper angles irrespective of morphology (ANCOVA: $F_{(1, 4)} = 2.42$; $P = 0.05$) and it is also the case that tree and shrub species tend to be smaller, up to an order of magnitude smaller, than subterranean or ground species.

Swimming: We found that the semi-aquatic species have the fastest absolute swimming velocities while the shrub species are the poorest swimmers (Figure 4.4, ANOVA with *Post Hoc* Tukey HSD: $F = 2.80$; Df = 4; $P = 0.05$). When size-corrected, semi-aquatic species remain the fastest swimmers while the other ecomorphs were slower (Figure 4.4, Table 8S). We also found a positive correlation between swimming velocity and hindlimb elements including tibiofibula, tarsus, and foot (Table 13S, 14S, 15S).

Principal Components Analysis: The first two principal component (PC) axes explain 88.4% of the total performance variation in the non-size corrected data (Figure 4.5; Table 4.1). PC1 correlated positively with cling angle and contrasted with jump distance and swimming velocity. PC 2 correlated positively with cling angle and jump distance. PC 3 correlated negatively with cling angle and swim velocity and explained 13% of the total performance variation. In the size-corrected PCA, the first two PC axes explain 87% of the variation (Figure 4.6; Table 4.1). PC1 correlated positively with cling angle and contrasted with swimming velocity. PC 2 correlated positively with jump distance and, to the lesser extent, swimming velocity and cling. Size-corrected PCA provided good separation of ecomorphs by performance ability. PC3 correlated positively with jump distance and contrasted with swim velocity and cling angle and also explained 13% of the variation.

Table 4.1. Loadings from principal components analyses of absolute (see Figure 4.5) and size-corrected (see Figure 4.6) performance *.

Variables	PC1	PC2	PC3
Absolute performance PC scores			
Cling angle	**0.59**	**0.478**	**-0.644**
Swim velocity	**-0.63**	-0.215	**-0.754**
Jump distance	**-0.49**	**0.852**	0.173
Percent variance explained	0.63	0.24	0.13
Size-corrected PC scores			
Cling angle	**0.706**	**0.377**	**-0.600**
Swim velocity	**-0.708**	**0.372**	**-0.600**
Jump distance	-0.003	**0.848**	**0.530**
Percent variance explained	0.46	0.41	0.13

*Note: Substantial loadings are marked in bold.

Canonical Correlation Analysis: Canonical correlation analysis between the size-adjusted morphology and size-adjusted performance variables indicate that two of the three canonical dimensions are statistically significant (Table 4.2). Dimension 1 had a canonical correlation of 0.90 between the sets of variables, while dimension 2 had a canonical correlation of 0.74. Below are the standardized canonical coefficients for the first two dimensions across both sets of variables (Table 4.3). The morphological variables load onto the first canonical dimension primarily as a tradeoff between the tibiofibula and toe pads (negative) versus the foot and tarsus (positive). These morphological variables correlated strongly with cling performance. Canonical correlation 2 describes an additional component of tradeoff between the tibiofibula (negative) and tarsus, which correlates with a tradeoff between cling performance (positive) and swim and jump performance (negative). These taken together indicate that swim and jumping performances are associated with morphologies that are more similar to each other than either is to cling performance.

Table 4.2. Tests of Canonical Dimensions

Dimension	Canonical Correlation	Multiple F	D.f. 1	D.f. 2	P
1	0.90	12.71	10	1635	7.49e-28
2	0.74	4.44	6	1186	2.90e-03
3	0.18	2.04	2	594	8.10e-02

Table 4.3. Standardized Canonical Coefficients

	Dimensions	
	1	2
Morphological Variables		
Toe-pad	-0.568	0.109
Hand	0.153	0.277
Tarsus	0.804	0.934
Tibiofibula	-1.702	-1.428
Foot	1.054	-0.376
Performance Variables		
Cling	-0.669	0.885
Swim	-0.338	-0.553
Jump	-0.203	-0.667

Evolutionary analyses on size-corrected performance

Evolutionary models fit to the size-adjusted performance data indicate that some of the performance capabilities are best explained by a model of divergent selection between microhabitat types while others are best explained by drift. The adaptive model, microhabitat use, best explained jump distance and swimming velocity while the drift model best explained cling angle (Table 4.4).

Table 4.4. Model fit statistics for all three size-corrected performance measurements. The model with the best fit, using AIC, is listed as 0, with ΔAIC values listed for all other models. Bootstrap model selection frequencies based on 2000 bootstrap replicates are included in parentheses.

Variables	Microhabitat	BM
Relative Clinging Performance	7.7 (57)	0 (43)
Relative Swimming Performance	0 (89)	3 (11)
Relative Jumping Performance	0 (93)	11 (7)

The parameter theta (Θ) is a predicted value of phenotypic optima and indicates evolution of performance differences among ecomorphs. For example, the semi-aquatic

species are evolving towards a large optimum for swimming velocity while ground and tree species are evolving towards an intermediate optimum and subterranean and shrub species are evolving towards a small swimming velocity optimum (Table 4.5).

Table 4.5. Parameter estimated for the microhabitat model for the performance variables. Strength of selection (α) and noise (σ) are shown for performance variables for which the microhabitat model performed best. Estimated optimal values (Θ) for each ecomorph are shown for body lengths swam and body lengths jumped. Only σ is displayed for cling angle as this variable was not explained by a selection-based model. Bootstrap 95% confidence intervals for parameter estimates are in parentheses.

	Relative cling	Relative swim	Relative jump
α		95.2 (96.4, 97.7)	36.3 (30.8, 41.9)
σ	0.41 (0.39, 0.44)	1.61 (1.54, 1.69)	7.66 (6.33, 8.98)
$\Theta_{Subterranean}$		1.32 (1.31, 1.34)	0.11(.06, 0.18)
Θ_{Ground}		1.43 (1.42, 1.44)	34000 (27000, 43000)
$\Theta_{Semi\text{-}aquatic}$		1.78, (1.75, 1.81)	5.31 (2.55, 11.7)
Θ_{Shrub}		1.36 (1.35, 1.36)	327 (263, 405)
Θ_{Tree}		1.39 (1.37, 1.40)	1200 (900, 1700)

Jump distance was also best explained by the microhabitat model and we find that ground species are evolving towards the largest optimum, shrub and tree species are evolving towards intermediate optimum for jump distance, and subterranean and semi-aquatic species are evolving towards small optimum (Table 4.5).

Cling angle performance as best explained by a model of neutral selection therefore, only sigma (σ) is shown (Table 4.5).

Discussion

We found that species have specializations for locomotor performance capabilities appropriate to their preferred microhabitats. For example, semi-aquatic

species excel at swimming, whereas ground species excel at jumping and arboreal species excel at clinging. These performance capabilities are associated with changes in morphology that facilitate these tasks, particularly in limb morphology. Jumping specialists have relatively long hindlimb elements, swimming species have elongated hindlimbs and paddle-like feet, and climbing species have large toe pads and elongated forelimbs, but shorter hindlimbs. Furthermore, our evolutionary analyses indicate that these differences have evolved via divergent selection in response to microhabitat and not drift.

Performance Capabilities and Microhabitat Use

Jumping: Jump distance is a function of take-off velocity and take-off angle alone (Equation 1). Once an animal is in the aerial phase of the jump it can no longer alter the distance jumped. We found that take-off velocity, and acceleration for that matter, do not differ between ecologies, therefore, jump angle is the only variable that species can alter to achieve varying jump distances (Equation 1). The lack of variation in take-off velocity has also been found by others (Altevogt et al. 1986; Marsh and John-Alder, 1994; Choi and Park, 1996). The ground ecomorph had the largest jump angle of all ecomorphs (~ 40°) and therefore the longest jump distance when corrected for body size. This need to increase the jump distance via maximizing the jump angle is necessary in the horizontal environment for predator avoidance. In contrast, the tree and shrub ecomorphs live in a horizontal and vertical environment and have the ability to utilize another dimension to avoid predators. Similarly, semi-aquatic species can jump into the stream to avoid predation. Given the limited dimensions ground species utilize, it is beneficial to maximize jump distance.

It is interesting that the take-off velocity did not differ between ecologies given their varying forms of locomotion. Emerson (1978) noted that this conservation of "quickness" in jumping velocity may be a key locomotor parameter across many frog species and may be important for predator avoidance. This may also be a conserved property of anuran musculature but it needs further testing

Clinging: Clinging performance is crucial for tree and shrub species as they utilize the tallest environments (Rivera and Butler, *in prep*). In frogs, the ability to stick to a

substrate is dependent on Stefan adhesion (Equation 2), a form of wet adhesion (Nachtigall, 1974; Emerson and Diehl, 1980; Hanna and Barnes, 1991). Emerson and Diehl (1980) found that viscosity and distance between discs do not vary across taxa therefore, these can be viewed as constants. Federle et al. (2006) also estimated a constant for surface tension (V) for mucus in their experiments. We did not take data on how long frogs were able to cling, but given that most variables are constants, toe-pad area (α) is the only variable that can differ to alter clinging performance. Indeed, the ability to cling to smooth surfaces is advantageous in an arboreal environment given that some individuals were caught at over a 30 m height.

Surprisingly, the semi-aquatic species were unable to cling to large angles despite having enlarged toe-pads. This result could be explained by use of a different clinging mechanism. This species was usually found clinging on rock walls in fast moving streams often on vertical faces of waterfalls. It is likely that this species is using an interlocking mechanism, and not Stefan adhesion, to cling to rough surfaces as described by Emerson and Diehl (1980). This mechanism is commonly used to grip to textured substrate like bark and rough rocks.

Swimming: It appears that the semi-aquatic species, *Austrochaperina palmipes*, has the fastest swim velocity compared to all other ecologies. It is also the case that they have the shortest swimming stroke cycle. This indicates that *A. palmipes* is able to cover more distance per stroke. Inversely, the shrub species had some of the fastest stroke cycles but slowest velocities. In fact, we found that *A. palmipes* produced some of the largest thrust values (Equation 3) compared to all other ecologies. How the feet produce this thrust force has been debated for several decades. Gal and Blake (1988 a,b) hypothesized that force was being produced by a central jet between the feet while others hypothesized a lift-based mechanism similarly used by birds as described by Johansson and Norberg (2003). Johansson and Lauder (2004) found no evidence of the central jet hypothesize using digital particle image velocimetry (DPIV) nor did they find evidence for a lift-based mechanism. Instead, Johansson and Lauder (2004) found that the mechanism is based on drag and acceleration reaction where thrust is being generated by vortex rings on the suction side of the feet. The vortices are shed behind the frog during the kicking phase of the swim cycle. These findings were also corroborated by Stamhuis

and Nauwelaerts (2005). This drag form of propulsion is common amongst semi-aquatic animals like turtles and crabs (Roper et al., 2011; Kim et al., 2013) and frogs with interdigital webbing (Nauwelaerts et al., 2005). Jizhuang et al. (2017) found that aquatic frogs with interdigital webbing had a higher propulsive efficiency (43%) when compared to ground species with no webbing (29.5%).

Size and Shape Variation

Jumping: We found that both absolute and relative jumping distances are size-dependent but have opposite relationships. Absolutely, large species jump farther distances than smaller species, but when corrected for SVL, smaller species outperform bigger species, although this is heavily driven by the subterranean species. This trend where jumping performance relative to SVL decreases with increased size has been noted by other authors as well (Emerson, 1978; Zug, 1978; Gomes et al., 2009), although great variation exists within and across species.

It is also that case that the ground species had the longest tibiofibula and foot elements of all ecomorphs (Chapter 3). This elongation of the hind limb for jumping performance is not well studied but Nauwelaerts et al. (2007) hypothesized that elongated foot elements provide high moments of inertia. Inertia is a product of mass and the square of perpendicular distance to the rotation. By having longer feet an individual frog will increase the moment of inertia and reduce foot ration . This allows the foot to remain in contact with the ground longer which allows for full extension of the leg during the jump and also contributes to jumping directly forward and not deviating from the trajectory. Nauwelaerts et al. (2007) also hypothesized that longer feet allow for more contact with the surface proving better traction and generation of thrust by reducing slippage.

Clinging: We found a negative correlation between clinging ability and mass (Figure 3C, D). Emerson and Diehl (1980) first described the relationship between size and clinging ability. All things being equal, smaller animals are able to cling to steeper angles when compared to larger species. Indeed, it is true that tree and shrub species are smaller than other ecologies and this miniaturization may be necessary for clinging. Miniaturization also seemed to be the reason why *Paedophryne* and some *Cophixalus*

species were able to cling to 180°, despite not having morphological adaptations that are common in climbing species, such as enlarged toe-pads. In contrast, increased mass improves clinging ability when using the interlocking mechanism (Emerson and Diehl, 1980). This appears to be the case in the semi-aquatic species as they are much larger than the tree and shrub species and performed poorly on the cling test, which was done a smooth surface, yet have enlarged toe-pads. It is doubtful that semi-aquatic species are using Stefan adhesion to cling as Stefan adhesion is disrupted by water and semi-aquatic species live in streams.

The tree and shrub ecomorphs also possess elongated forelimbs when corrected for size. Although these morphologies are not necessarily related to clinging performance, they do seem to play an important role in climbing. It is important to note that only tree and shrub species perform these two activities, clinging and climbing. This elongation is correlated with the elongation of climbing musculature like the extensores breves profundi and the presence of the extensores breves distalis in the forearm (Burton, 1998). Furthermore, Manzano et al. (2008) showed that a shrub and tree species of frogs used their forelimbs and hands to traverse narrow dowel rods and are able to perform maneuvers that require fine control of the forelimbs such as grasping the dowel. This control of the forelimbs is atypical of frogs that use other forms of locomotion. Arboreal species that possess elongated, well-developed forelimbs are even capable of complex movements like manipulating prey items while feeding (Gray et al. 1997).

Swimming: The sole semi-aquatic species in this study, *A. palmipes*, differed from all other species in that it was dorsally compressed. A flat profile is typical of aquatic species and aids in swimming by being more streamlined. A more streamlined shape allows for laminar flow reducing pressure drag and thereby turbulence in the wake of the frog.

Similar to jumping, we found that semi-aquatic animals have elongated feet. Nauwelaerts et al. (2005) showed that the foot elongation is used in a same fashion as jumping where it increases the moment of inertia. This allows the foot the withstand any rotation of the kicking phase in the swim cycle. It is crucial for a semi-aquatic animal to withstand this force in order to stay perpendicular to the flow and be able to produce

thrust and propel itself forward. The elongation of the foot also provides a larger surface area, like a paddle, than can be used for thrust.

Evolution of performance and trade-offs

Our evolutionary analysis demonstrates that two of the three performance axes are strongly shaped by adaptive evolution. Relative jump distance and relative swimming velocity are evolving in response to microhabitat use, whereas clinging ability can be best explained by neutral evolution. The best jumpers after correcting for body size were the ground-dwelling frogs, followed by tree and shrub species, then semi-aquatic species and finally subterranean species. Whereas the best swimmers follow a different order: the semi-aquatic species were best by far, followed by ground, tree, shrub, and subterranean species. It is interesting that the ground species were the best or second best at both performances, whereas the semiaquatic species was below average at jumping. We also found that swimming and jumping performance covary and this performance also varies with tibiofibula and foot length (Table 4.3).

Jumping and swimming are two performance abilities in frogs that rely heavily on the hind limbs morphology, particularly foot length, and musculature. Both ground and semi-aquatic ecomorphs possess an elongated foot compared to other ecomorphs and it appears that similar kinematics are involved in both performances. The primary the role of the elongated foot in jumping and swimming is force production against the ground or water, respectively (Nauwelaerts and Aerts, 2003). Indeed, we found that terrestrial species were good swimmers compared to other non-aquatic ecomorphs. This is was supported by the estimated optimal values of theta in our evolutionary analysis. Both ground and semi-aquatic ecomorphs had large optima for the swimming performance compared to other ecomorphs.

However, if this were the complete explanation, semi-aquatic species would also be able to jump distances comparable to ground species, which was not the case. Instead, semi-aquatic species were some of the poorest jumpers when corrected for body size. This mismatch in performance between the ground and semi-aquatic species may be due to selective pressure of predator escape response. Ground species possess the classic morphology optimized for saltatory locomotion, with high take-off angles, which allows

ground species to jump farther to escape from predators or curious researchers. On the other hand, semi-aquatic species do not need to maximize jump distances as their predator escape response is to fall or hop from rocks where they perch into the stream. This is also supported by our estimated optimal values of theta for jump distance as ground species had large thetas while semi-aquatic species had small theta values.

Clinging ability is heavily influenced by mass as the strength of clinging must support the animal's mass against the pull of gravity. Therefore, smaller animals have an inherent advantage as they can cling upside down even without morphological adaptations. Inversely, larger animals, like *C. doriae* are unable to cling even at small angles. This is because surface area is proportional to mass$^{2/3}$, so as animals increase in surface area (size), they also must increase in mass.

We do find a trade-off between clinging and swimming. It appears that species cannot maximize both performance tasks as species that cling at steep angles cannot swim at fast velocities and vice versa. This may be a consequence of morphology as tree and shrub species tend to have shortened hindlimb elements, which are disadvantages for swimming. Moreover, we also find a conflict between maximizing jumping distance and clinging performance. Ground species that maximize their jump distances have poor clinging ability, likely due to the lack of morphological adaptations associated with clinging. However, species that excel at clinging are not necessarily poor jumpers. Tree and shrub species had somewhat comparable jump distances to ground species when corrected for body size. This may also be a consequence of predator escape response as shrub species can be found on the ground at times and may need to escape predation by jumping far distances. It is not clear why tree species also jump long distances but it may be used to jump from branch to branch, although little information is known about the ecology of the tree ecomorph.

Conclusion

We find that species excel at performance tasks that are most relevant to their habitat and significantly differ between ecomorphs. Coupled with these performance differences are specialized morphologies that allow ecomorphs to perform these tasks. We also find trade-offs exist between performance capabilities so that species cannot

maximize all three tasks, but many species can accomplish two. Last, jumping and swimming performance seem to be evolving in response to selection for habitat but clinging performance is not.

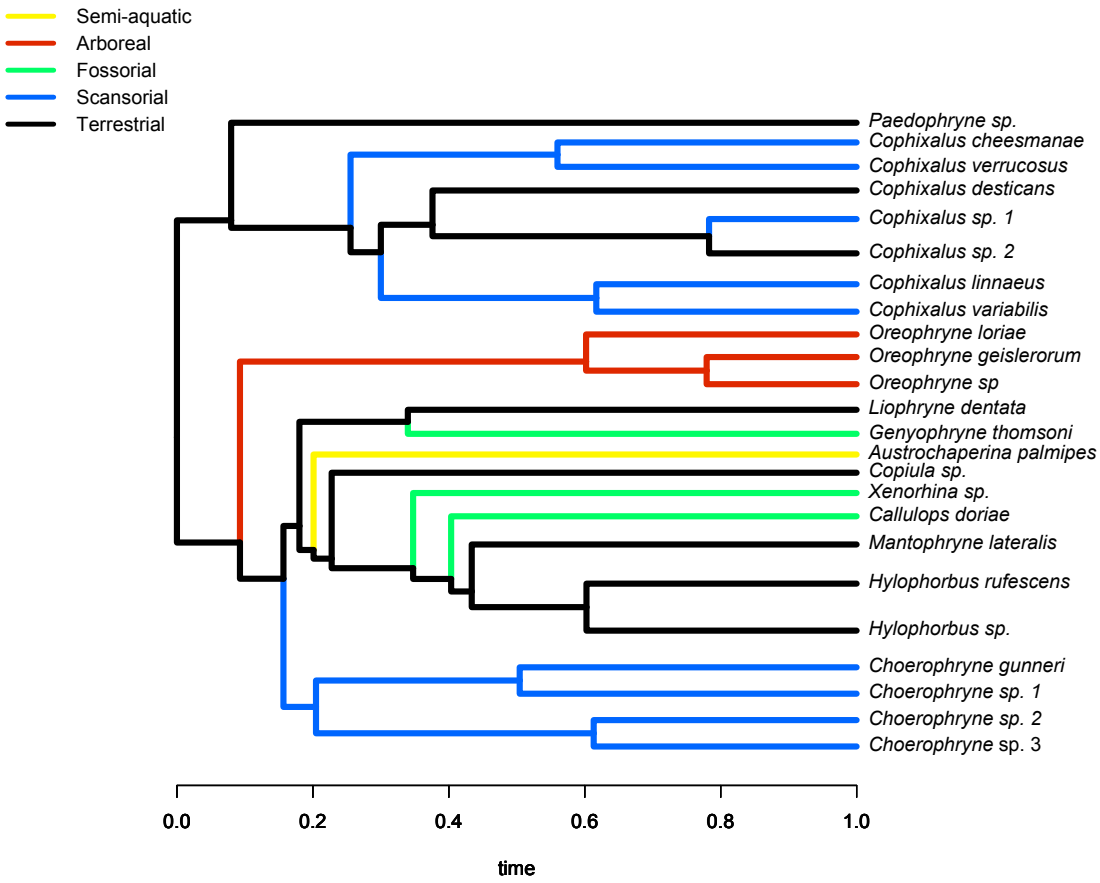


Figure 4.1. The *Asterophyinae* phylogeny used in this study with branch lengths proportional to time (modified from Rivera et al., 2017). Microhabitats are mapped onto the phylogeny represented by different colors.

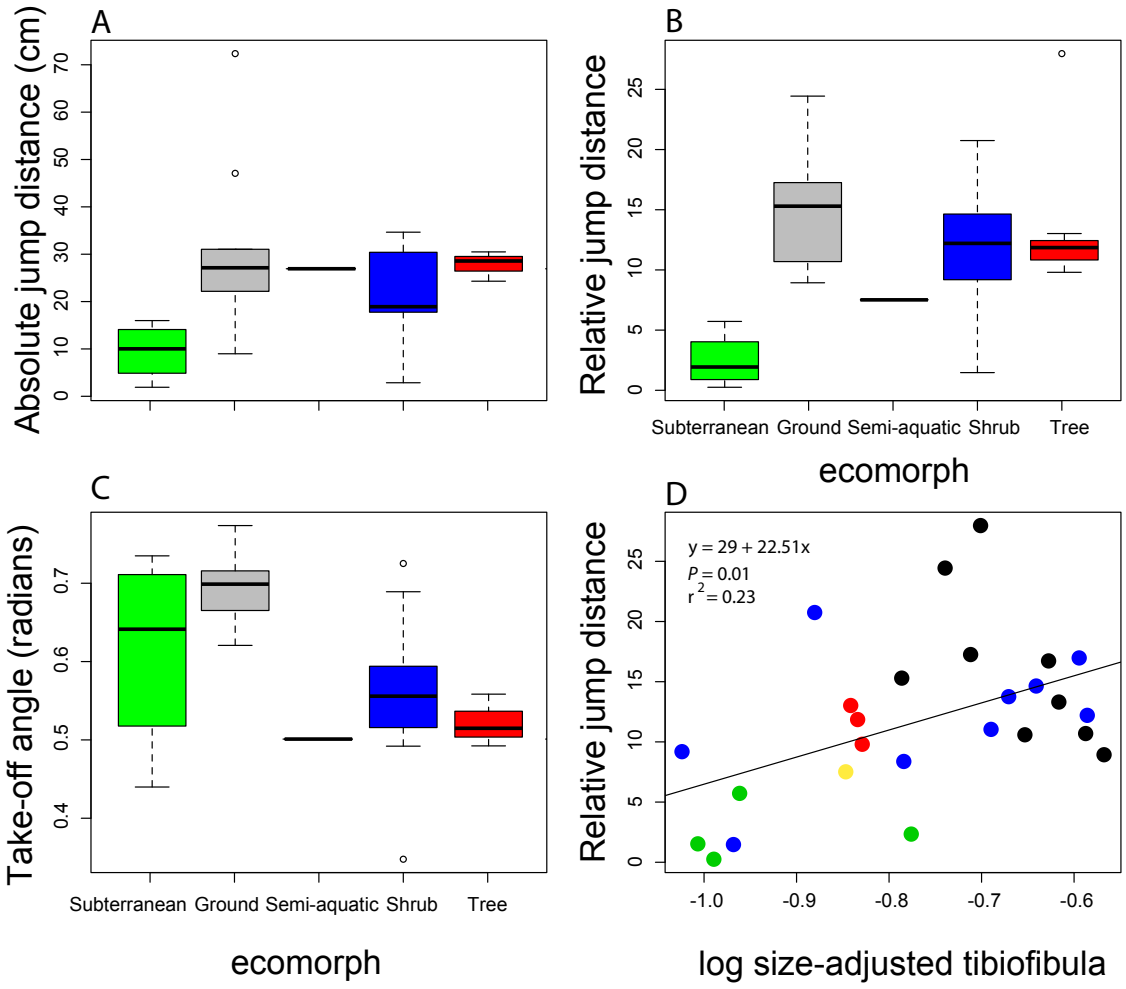


Figure 4.2. Jumping performance species means grouped by ecomorph for absolute jump distance (A), size corrected jump distance (B), take-off angle (C), and a linear regression for size corrected jump distance as a function of log transformed, size-corrected tibiofibula length (D).

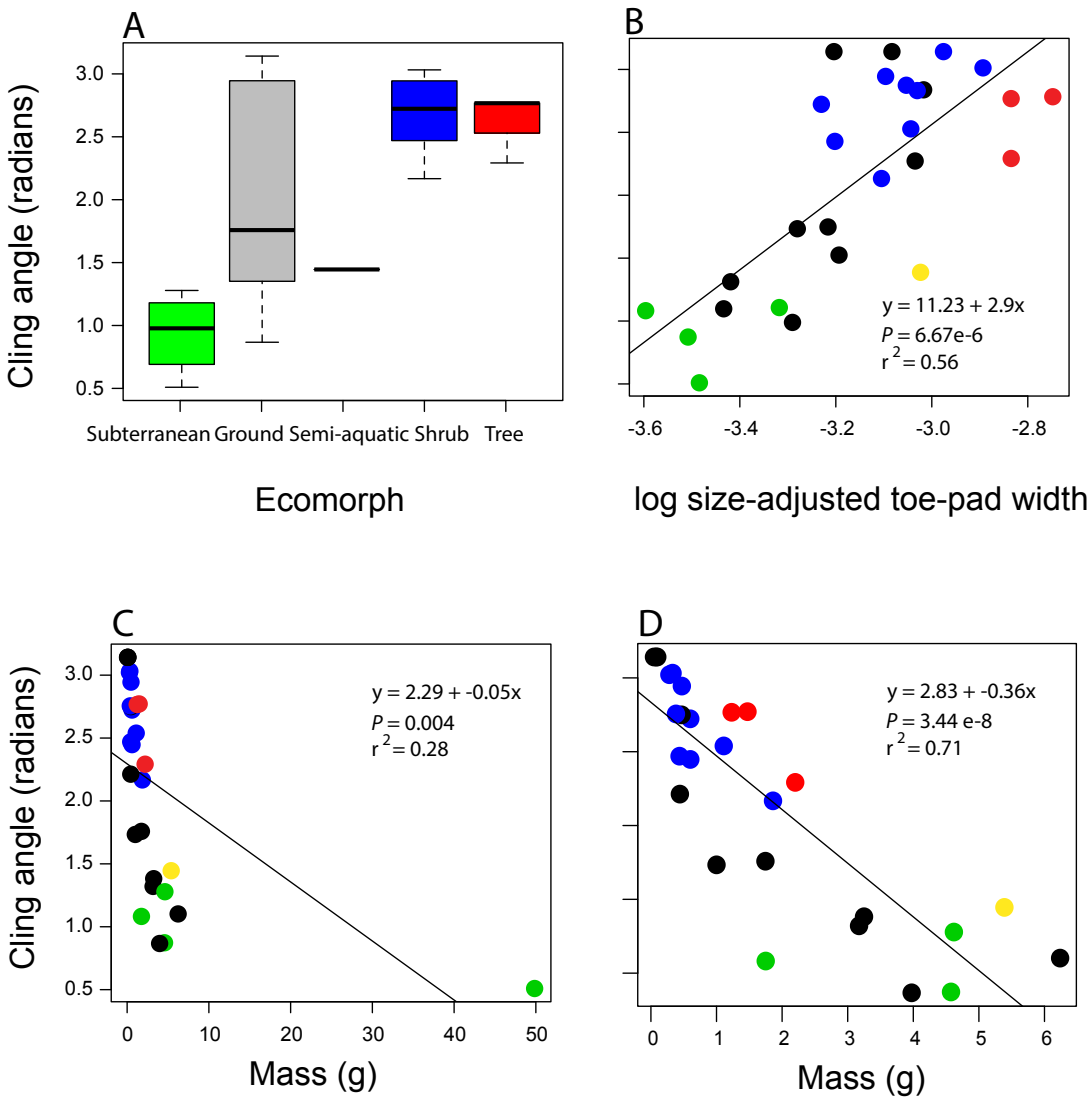


Figure 4.3. Clinging performance species means grouped by ecomorph (A), linear regression of clinging performance as a function of size-adjusted toe-pad width (B), a linear regression of clinging performance as a function of mass (C), and a linear regression of clinging performance as a function of mass with *C. doriae* removed (D).

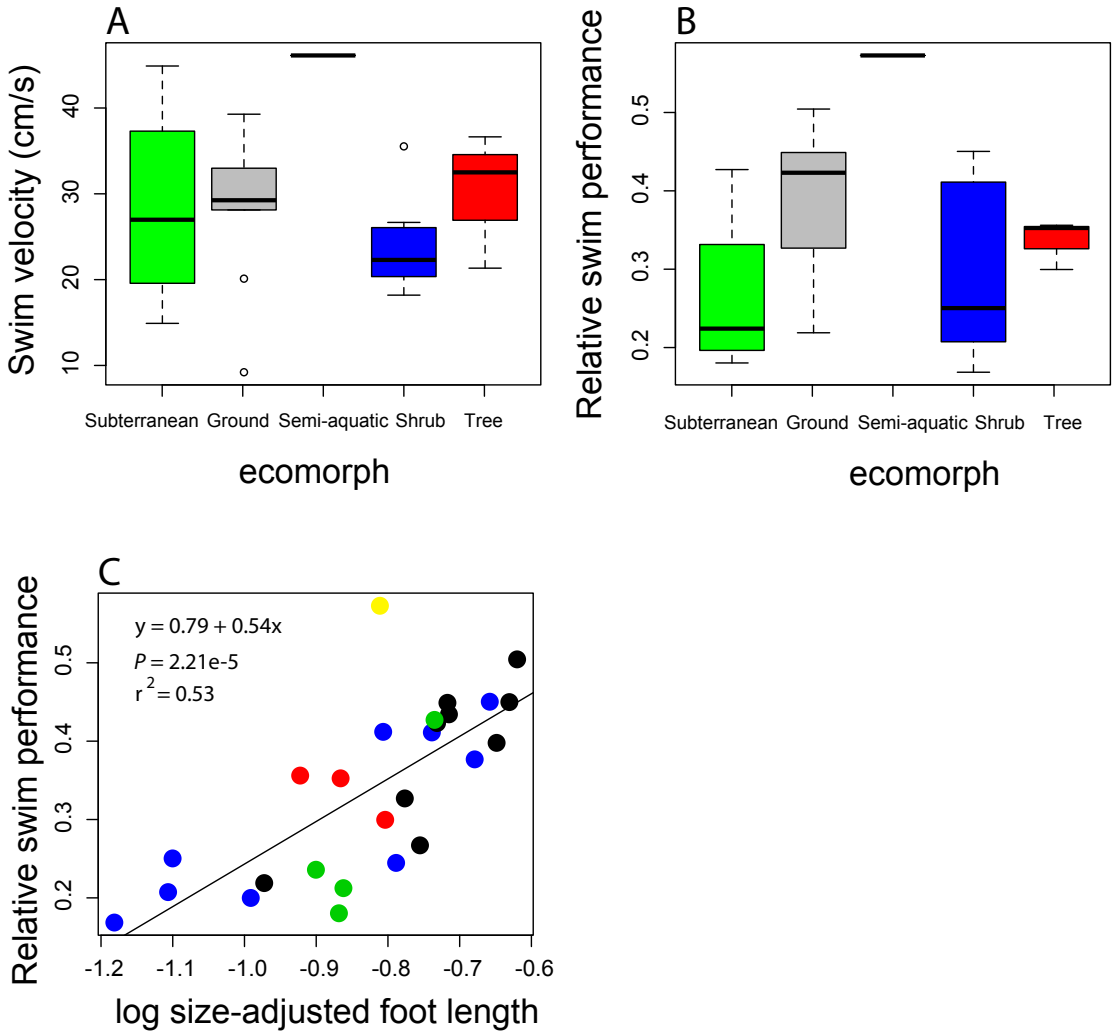


Figure 4.4. Swimming performance species means grouped by ecomorph. (A) Absolute swimming velocity is shown on the top left and (B) relative swimming velocity is shown on the top right. A linear regression of relative swimming velocity as a function of size-adjusted foot length is shown in (C).

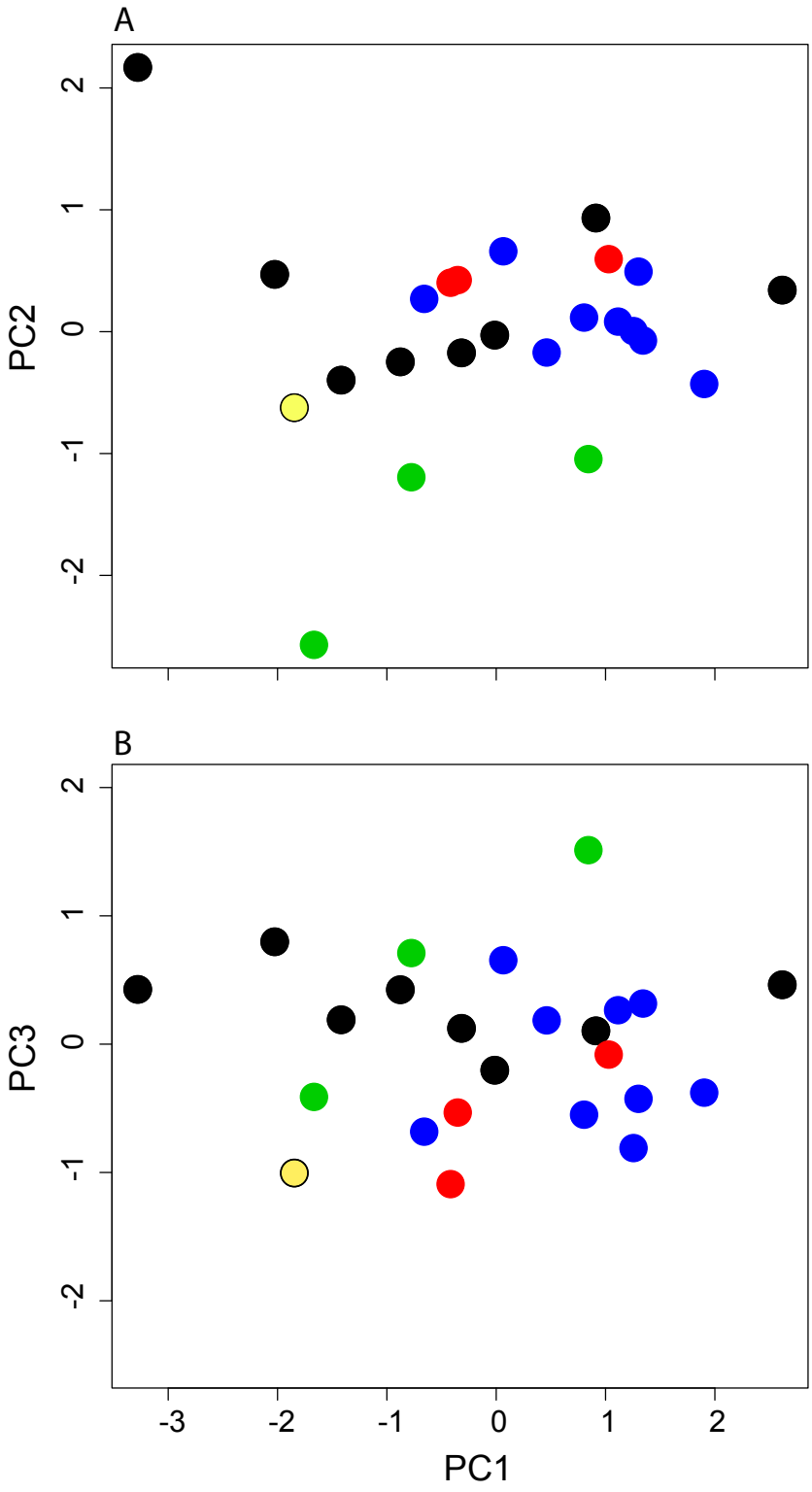

A
PC2
2
1
0
-1
-2
B
PC3
2
1
0
-1
-2
-3
-2
-1
0
1
2
PC1

Figure 4.5. Plot of principal component scores for absolute performance of cling angle, swimming velocity, and jumping distance for PC1 vs PC2 (A) and PC1 vs PC3 (B). Colors indicate ecomorph type where green = subterranean, blue = shrub, yellow fill = semi-aquatic, black = ground, and red = tree.

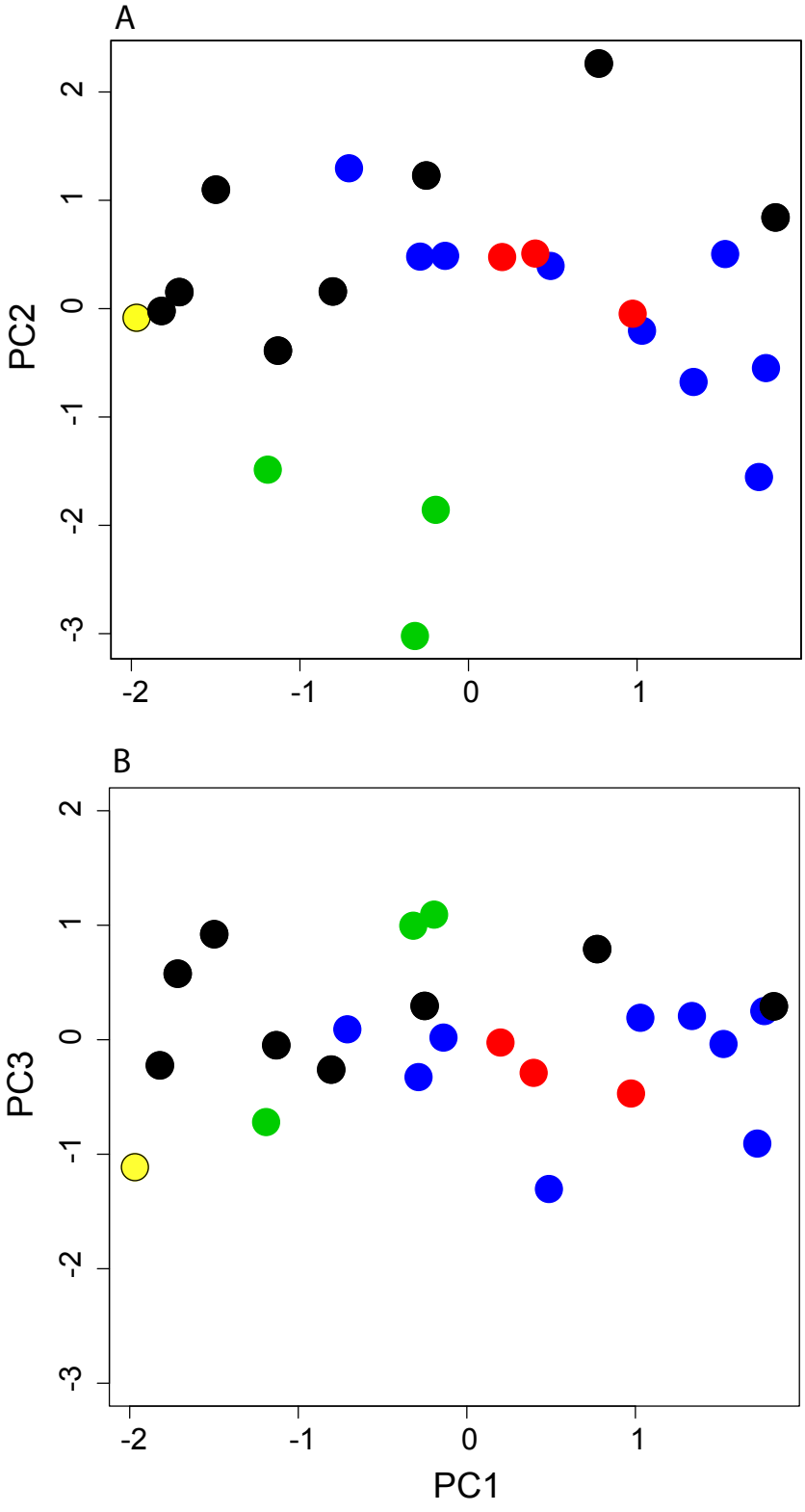

A
PC2
2
1
0
-1
-2
-3
-2
-1
0
1
B
PC3
2
1
0
-1
-2
-3
-2
-1
0
1
PC1

Figure 4.6. Size corrected PCA for jump distance, swimming velocity, and cling angle for PC1 vs PC2 (A) and PC1 vs PC3 (B). Colors indicate ecomorph type where green = subterranean, blue = shrub, yellow fill = semi-aquatic, black = ground, and red = tree.

APPENDIX

Table 1S. List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Species	BPBM	Localities
Albericus brunhildae	FK11830	Torricelli Mts, West Sepik Province, PNG
Albericus darlingtoni	33664	Mt. Itukua, Southern Highlands, PNG
Albericus exclamitans	18317	Mt. Shungol, Morobe Province, PNG
Albericus gudrunae	35561	Madang Proince, PNG
Albericus gunnari	18351	Dorobisoro, Central Province, PNG
Albericus murritus	33637	Southern Highlands Province, PNG
Albericus sanguinopictus	17847	Etakaba Creek
Aphantophryne pansa	AA21610	
Aphantophryne pansa	AA21608	
Aphantophryne pansa	AA21612	
Aphantophryne pansa	AA21609	
Asterophrys leucopus	28153	Moran Rd.
Asterophrys turpicola	28204	Libano
Austrochaperina basipalmata	22665	Torricelli Mts, West Sepik Province, PNG

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Austrochaperina blumi	22656	Torricelli Mts, West Sepik Province, PNG
Austrochaperina guttata	13157	Lakekamu
Austrochaperina macrorhyncha	13862	Timika Airport
Austrochaperina novaebritanniae	22481	Nakanai Mts, New Britain Province, PNG
Austrochaperina palmipes	15234	Cloudy Mts.,
Austrochaperina parkeri	18388	Mt. Shungol, Morobe Province, PNG
Austrochaperina blumi	35735	Mindangua Stream, East Sepik Province, PNG
Austrochaperina septentrionalis	22668	Torricelli Mts, West Sepik Province, PNG
Austrochaperina sp. 1	22664	Torricelli Mts, West Sepik Province, PNG
Austrochaperina sp. 3	40000	Mt. Trafalgar, Oro Province, PNG
Austrochaperina yelaensis	20112	Mt. Rossel, Rossel Island, Milne Bay Province, PNG
Barygenys atra	25756	Suzuki Track, Morobe Province
Barygenys exsul	20126	Mt. Rio, Sudest Island, Milne Bay Province, PNG
Barygenys exsul	20128	Rossel Islands, Milne Bay Province, PNG
Barygenys maculata	38914	

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Barygenys sp. 1	25760	Duabo, Pini Range, Milne Bay Province, PNG
Barygenys apodasta	38904	Woodlark Islands, Milne Bay Province, PNG
Callulops doriae	20141	
Callulops eremnosphax	13155	Lakekamu
Callulops microtis	35836	Samorek, Madang Province, PNG
Callulops personatus	18504	Mt. Shungol, Morobe Province, PNG
Callulops robustus	16806	Bwaga Bwaga Ridge, Misima Island, Milne Bay Province, PNG
Callulops sp. 1	37122	Mt. Victory, Oro Province, PNG
Callulops sp. 1 (doriae)	19236	
Callulops wilhelmanus	33669	Mt. Itukua, Southern Highlands Province, PNG
Choerophryne longirostris	22675	Torricelli Mts, West Sepik Province, PNG
Choerophryne proboscidea	34679	Mindangua Stream, East Sepik Province, PNG
Choerophryne rostellifer	22685	Torricelli Mts, West Sepik Province, PNG
Choerophryne bryonopsis	39991	Mt. Trafalgar, Oro Province, PNG
Cophixalus albolineatus	18430	Mt. Shungol, Morobe Province, PNG

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Cophixalus albolineatus	18429	Mt. Shungol, Morobe Province, PNG
Cophixalus ateles	19314	
Cophixalus balbus	22692	Torricelli Mts, West Sepik Province, PNG
Cophixalus caverniphilus	33707	Mt. Itukua, Southern Highlands Province, PNG
Cophixalus cheesmanae	18392	Mt. Shungol, Morobe Province, PNG
Cophixalus clapporum	37718	Woodlark Islands, Milne Bay Province, PNG
Cophixalus cryptotympanum	17959	Etakaba Creek
Cophixalus cupricarenus	20215	Rossel Islands, Milne Bay Province, PNG
Cophixalus daymani	39048	Milne Bay Province, PNG
Cophixalus desticans	15708	Mt. Pekopekowana, Milne Bay Province, PNG
Cophixalus iovaorum	19277	
Cophixalus melanops	20197	Mt. Rio, Sudest Island, Milne Bay Pr
Cophixalus nexipus	19320	
Cophixalus pipilans	35843	East Sepik Province, PNG
Cophixalus takinesa (undescribed)	15707	Mt. Pekopekowana, Milne Bay Province, PNG

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Cophixalus timidus	18097	Mt. Simpson, Central Province, PNG
Cophixalus variabilis	15814	Mt. Pekopekowana, Milne Bay Province, PNG
Cophixalus verrucosus	27491	Central Province, PNG
Copiula fistulans	18605	Mt. Shungol, Morobe Province, PNG
Copiula minor	15665	Cloudy Mts.,
Copiula oxyrhina	17084	Bwaga Bwaga, Misima Island, Milne Bay Province, PNG
Copiula sp. 3	20262	Sudest Islands, Milne Bay Province, PNG
Copiula sp. 5	39007	Woodlark Island, Milne Bay Province, PNG
Copiula sp. 6	38939	Milne Bay Province, PNG
Copiula sp. 7	40083	Mt. Trafalgar, Oro Province, PNG
Copiula sp. nov. 1	20287	Rossel Island, Milne Bay Province
Copiula sp. nov. 2	17827	
Copiula tyleri	22708	Torricelli Mts, West Sepik Province, PNG
Dyscophis antongilli	39559	
Genyophryne thomsoni	20357	Sudest Island, Milne Bay Province, PNG

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Hylophorbus extimus	20369	Sudest Island, Milne Bay Province,PNG
Hylophorbus macrops	22740	Torricelli Mts, West Sepik Province, PNG
Hylophorbus myopicus	39638	Woodlark Island, Milne Bay Province, PNG
Hylophorbus proekes	22761	Torricelli Mts, West Sepik Province, PNG
Hylophorbus richardsi	33749	Southern Highlands Province, PNG
Hylophorbus atrifasciatus	34724	Mindangua Stream, East Sepik Province, PNG
Hylophorbus sp. 1(rufescens)	15355	Duabo, Pini Range, Milne Bay Province, PNG
Hylophorbus sp. 11	18450	Mt. Shungol, Morobe Province, PNG
Hylophorbus sp. 12	19338	
Hylophorbus sp. 13	22504	Central Province, PNG
Hylophorbus sp. 14	35986	Mindangua Stream, East Sepik Province, PNG
Hylophorbus sp. 15	35866	Madang Province, PNG
Hylophorbus sp. 16	37232	Oro Province, PNG
Hylophorbus sp. 2	35692	Madang Province, PNG
Hylophorbus sp. 2	18033	

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Hylophorbus sp. 3	39569	Milne Bay Province, PNG
Hylophorbus sp. 3	18441	Dorobisoro, Central Province, PNG
Hylophorbus sp. 7	16181	Fergusson Island, Milne Bay Province, PNG
Hylophorbus sp. 8	17048	Bwaga Bwaga, Misima Island, Milne Bay Province, PNG
Liophryne allisoni	18315	
Liophryne dentata	15373	Cloudy Mts.,
Liophryne magnitympanum	19360	
Liophryne schlaginhaufeni	22754	Torricelli Mts, West Sepik Province, PNG
Liophryne miniafia	39929	Mt. Trafalgar, Oro Province, PNG
Mantophryne axanthogaster	20397	Sudest Island, Milne Bay Province, PNG
Mantophryne lateralis	19265	Laronu
Mantophryne louisiadensis	20142	Rossel Island, Milne Bay Province, PNG
Mantophryne sp. 1	15410	Cloudy Mts.,
Mantophryne sp. 2	20396	Normanby Island, Milne Bay Province, PNG
Mantophryne sp. 3	22780	Torricelli Mts, West Sepik Province, PNG

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Mantophryne sp. 4	40135	
Metamagnusia marani	6303	
Metamagnusia marani	6304	
Metamagnusia marani	6305	
Metamagnusia slateri	13110	
Oninia senglaubi	6298	
Oreophryne anamiatoi	33763	Southern Highlands Province, PNG
Oreophryne annulata	1366 (KU)	Philippines
Oreophryne biroi	34688	Mindangua Stream, East Sepik Province, PNG
Oreophryne brachypus	22511	New Britain
Oreophryne ezra	20469	Mt. Rio, Sudest Island, Milne Bay Province, PNG
Oreophryne geislerorum	18510	Mt. Shungol, Morobe Province, PNG
Oreophryne inornata	16217	Mt. Kilkerran, Fergusson Island, Milne Bay Province, PNG
Oreophryne insulana	16546	Mt. Kilkerran, Fergusson Island, Milne Bay Province, PNG
Oreophryne loriae	22539	Central Province

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Oreophryne nana	11118 (KU)	Philippines
Oreophryne notata	33674	Southern Highlands Province, PNG
Oreophryne parkeri	22784	West Sepik Province, PNG
Oreophryne rossel	20571	Mt. Rossel, Rossel Island, Milne Bay Province, PNG
Oreophryne rossel	20572	Mt. Rossel, Rossel Island, Milne Bay Province, PNG
Oreophryne rossel	20538	Mt. Rossel, Rossel Island, Milne Bay Province, PNG
Oreophryne sp. 1	15775	Cloudy Mts.,
Oreophryne sp. 10	20522	Rossel Island, Milne Bay Province, PNG
Oreophryne sp. 11	20532	Mt. Rossel, Rossel Island, Milne Bay Province, PNG
Oreophryne sp. 12	22689	Torricelli Mts, West Sepik Province, PNG
Oreophryne sp. 14	34677	Madang Province, PNG
Oreophryne sp. 15	39510	Woodlark Island, Milne Bay Province, PNG
Oreophryne sp. 2	18128	Bwaga Bwaga, Misima Island, Milne Bay Province, PNG
Oreophryne sp. 3	16554	Normanby Islands, Milne Bay Province, PNG
Oreophryne sp. 3	18002	

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Oreophryne sp. 4	17980	
Oreophryne sp. 5	22789	Torricelli Mts, West Sepik Province, PNG
Oreophryne sp. 6	18509	
Oreophryne sp. 7	19371	
Oreophryne sp. 8	20440	Sudest Island, Milne Bay Province, PNG
Oreophryne sp. 9	20501	Sudest Island, Milne Bay Province, PNG
Oreophryne suckling 1	39244	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 2	39000	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 3	39366	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 4	39344	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 5	39357	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 6	38988	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 7	38992	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 8	38990	Mt. Suckling, Milne Bay Province, PNG
Oreophryne suckling 9	39156	Mt. Suckling, Milne Bay Province, PNG

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Oreophryne trafalgar 1	40143	Mt. Trafalgar, Oro Province, PNG
Oreophryne trafalgar 2	40145	Mt. Trafalgar, Oro Province, PNG
Oreophryne variabilis	1310 (KU)	Sulawesi
Oxydactyla crassa	17890	Mt. Simpson, Central province, PNG
Paedophryne sp.	FK16196	Mt. Trafalgar, Oro Province, PNG
Paedophryne swiftorum	31879	
Paedophryne verrucosa	FK15527	Milne Bay Province
Mantophryne menziesi	31477	Central Province, PNG
Platypelis grandis	37973	
Pseudocallulops eurydactylus	6300	
Scaphioprhyne marmorata	54292	
Sphenophryne cornuta	22793	Torricelli Mts, West Sepik Province, PNG
Xenorhina adisca	21474	
Xenorhina arboricola	22797	Torricelli Mts, West Sepik Province, PNG
Xenorhina mehelyi	28179	

Table 1S. (Continued) List of species and their corresponding catalog numbers from the Bernice Bishop Museum, Honolulu, HI, USA.

Xenorhina obesa	34741	Madang Province, PNG
Xenorhina ocellata	14060	
Xenorhina oxycephala	34697	East Sepik Province, PNG
Xenorhina parkerorum	33780	Southern Highlands Province, PNG
Xenorhina tumulus	22795	Torricelli Mts, West Sepik Province, PNG

Table 2S. Summary of taxonomic revisions based on our study.

Current Generic Taxonomy	Suggested Generic Taxonomy	Species
Aphantophryne	Aphantophryne	No Change
Asterophrys Metamagnusia Pseudocallulops	Asterophrys	*Asterophrys turpicola* *Asterophrys leucopus* *Metanagnusia slateri* *Metamagnusia marani* *Pseudocallulops eurydactylus*
Austrochaperina	Austrochaperina	No Change
Barygenys	Barygenys	No Change
Callulops	Callulops	No Change
Choerophryne	Choerophryne	No Change
Cophixalus	Cophixalus	No Change
Copiula	Copiula	No Change
Hylophorbus	Hylophorbus	No Change
Liophryne Genyophryne Oxydactyla Sphenophryne	Liophryne	*Liophryne allisoni* *Liophryne dentata* *Liophryne schlaginhaufeni* *Liophryne magnitympanum* *Liophryne sp. 1* *Genyophryne thomsoni* *Oxydactyla crassa* *Sphenophryne cornuta*

Table 2S. (Continued) Summary of taxonomic revisions based on our study.

Mantophryne	Mantophryne	No Change
Oninia	Oninia	No Change
Oreophryne	Oreophryne	No Change
Paedophryne	Paedophryne	No Change
Xenorhina	Xenorhina	No Change

Table 3S. Species, ecomorph, perch height (cm), perch diameter (cm), and mean morphology (mm).

species (N)	Perch height	Perch diameter	SVL	Femur	Tibiofibula	Tarsus	Foot	Head length	Head width	Humerus	Radioulna	Hand	Toe pad width
Tree													
Oreophryne geislerorum (3)	211.50	3.50	23.95	10.21	10.24	6.58	9.89	6.88	8.41	6.35	5.34	5.76	1.42
Oreophryne loriae (18)	322.91	8.91	24.56	10.34	10.52	6.84	9.94	6.34	8.38	6.25	5.30	6.36	1.51
Oreophryne sp. (9)	274.31	6.34	24.93	10.71	10.92	7.09	10.76	7.43	8.64	6.91	5.65	7.12	1.60
Subterranean													
Barygenys atra (4)	-0.25	NA	22.20	9.30	8.71	5.91	9.07	7.32	9.25	5.27	3.73	4.43	0.61
Callulops doriae (7)	-8.0	14	76.01	32.20	28.80	17.97	30.89	14.16	25.45	19.24	14.55	18.48	2.28
Callulops personatus (1)	0.01	NA	79.80	33.31	29.16	19.37	32.39	17.72	26.43	21.16	15.46	17.62	2.12
Genyophryne thomsoni (20)	-0.90	3.50	31.46	13.41	11.32	7.71	13.42	9.97	15.09	8.02	5.93	6.87	0.99
Xenorhina sp. (7)	-1.89	4.50	32.24	14.36	14.50	9.11	14.79	8.19	11.74	8.43	6.09	7.00	1.16

Table 3S. (Continued) Species, ecomorph, perch height (cm), perch diameter (cm), and mean morphology (mm).

Shrub													
Choerophryne gunneri (22)	92.92	5.37	16.36	7.53	7.28	4.75	6.01	4.67	5.39	5.18	4.39	5.06	0.80
Choerophryne sp. 1 (2)	0.01	NA	20.11	8.28	7.18	4.89	5.43	7.40	5.58	5.79	4.82	4.64	0.80
Choerophryne sp. 2 (7)	209.85	3.84	16.24	7.08	6.82	4.39	5.53	4.26	5.42	4.75	3.70	4.54	0.77
Choerophryne sp. 3 (6)	121.95	6.60	15.38	6.78	6.27	3.77	5.24	4.68	5.47	4.74	3.60	4.32	0.82
Cophixalus cheesmanae (16)	124.15	7.02	26.70	13.26	14.47	8.82	12.71	8.89	9.20	7.20	6.02	7.02	1.24
Cophixalus sp. 1 (23)	132.07	4.86	16.43	8.02	8.23	5.14	7.73	5.06	5.72	4.96	4.03	4.65	0.66
Cophixalus linnaeus (1)	28.60	5.00	13.25	6.86	6.98	4.08	5.94	4.19	4.66	3.56	2.80	3.18	0.80
Cophixalus variabilis (45)	108.66	2.98	13.76	6.80	7.06	4.24	6.42	3.69	4.79	3.79	3.03	3.70	0.67
Cophixalus verrucosus (30)	77.39	4.38	19.91	10.41	10.86	6.51	10.22	6.31	7.10	5.41	4.66	5.53	0.95
Semi-aquatic													
Austrochaperina palmipes (60)	52.24	66.17	35.29	16.91	15.28	9.41	15.81	11.31	12.61	9.49	7.15	9.46	1.78
Austrochaperina palmipes (60)	52.24	66.17	35.29	16.91	15.28	9.41	15.81	11.31	12.61	9.49	7.15	9.46	1.78

Table 3S. (Continued) Species, ecomorph, perch height (cm), perch diameter (cm), and mean morphology (mm).

Ground													
Cophixalus disticans (37)	12.73	5.34	13.24	6.57	7.00	4.17	6.69	3.87	4.59	4.02	2.76	2.95	0.63
Cophixalus sp. 2 (10)	58.03	4.79	12.27	5.94	6.12	3.37	5.54	3.73	4.81	3.92	2.63	3.16	0.61
Cophixalus pipilans (3)	0.01	NA	19.53	9.49	9.96	6.09	9.17	7.19	6.94	4.79	4.35	4.27	0.74
Copiula sp. (20)	0.01	NA	23.36	11.57	12.17	7.60	11.71	7.31	8.44	6.86	5.32	5.57	0.90
Hylophorbus rufscens (38)	13.53	50.66	31.86	15.48	17.03	10.22	16.29	9.82	10.97	9.66	7.96	8.30	1.05
Hylophorbus sp. (43)	21.38	10.16	34.77	17.36	18.74	11.21	18.51	10.23	11.70	10.36	8.67	9.36	1.29
Liophryne dentata (23)	4.30	1.50	27.34	14.71	15.26	9.59	14.70	8.98	11.56	7.96	6.60	6.36	1.17
Mantophryne lateralis (39)	19.75	5.50	40.44	18.84	19.62	12.22	19.67	12.47	14.71	11.63	9.36	10.20	1.34
Paedophryne sp. 1 (2)	0.01	NA	6.25	2.97	2.81	1.83	2.43	1.81	2.38	2.24	1.40	1.14	0.30
Paedophryne sp. 2 (5)	0.01	NA	7.28	3.56	3.60	2.24	2.87	1.98	2.84	2.28	1.66	1.16	0.30
Paedophryne sp. 3 (7)	7.5	7.0											

Table 4S. Performance and SVL means of species separated by ecomorph. Units are located under each heading.

Species (N)	Snout-vent length (mm)	Mass (g)	Jump distance (cm)	Relative jump distance	Jump angle	Jump velocity (m/s)	Jump acceleration (m/s^2)	Jump force (mN)	Max cling angle	Relative cling (radians)	Swim velocity (cm/s)	Relativ swim velocity
Subterranean												
Callulops doriae (7)	76.38	49.85	1.91	1.53	25.20	0.58	4.16	244	29.17	0.50	44.89	0.18
Genyophryne thomsoni (20)	29.24	4.30	15.99	5.72	34.12	1.53	10.93	45.0	50	0.87	29.71	0.21
Xenorhina sp (7)	33.50	5.01	7.83	2.34	39.37	1.53	10.99	29.2	60.75	1.10	14.90	0.43
Ground												
Cophixalus disticans (37)	13.46	0.48	22.95	17.28	40.50	1.87	13.33	6.27	121.30	2.27	30.57	0.40
Cophixalus sp. 2 (10)	12.71	0.35	29.24	23.28	40.72	1.82	13.02	4.23	157.54	2.84	20.11	0.33
Copiula sp. (20)	23.25	1.83	24.21	10.96	39.13	2.17	15.47	29.0	100.74	1.74	28.92	0.43
Hylophorbus rufscens (38)	30.40	3.25	28.27	9.11	39.66	2.06	14.73	49.9	75.67	1.31	30.36	0.42
Hylophorbus sp. (43)	35.25	4.20	47.22	13.28	42.51	2.86	20.41	88.7	49.67	0.98	33.25	0.45

Table 4S. (Continued) Performance and SVL means of species separated by ecomorph. Units are located under each heading.

Liophryne dentata (23)	27.55	3.00	29.78	10.27	35.76	2.13	15.20	50.2	64.79	1.52	37.86	0.50
Mantophryne lateralis (39)	40.65	6.56	77.69	18.46	38.43	2.62	18.72	153.0	63.13	1.09	39.78	0.44
Paedophryne sp. 1 (2)	6.11	0.11	9.68	15.51	38.71	1.24	12.02	1.20	180	3.14	9.20	0.21
Semi-aquatic												
Austrochaperina palmipes (60)	36.53	5.89	26.93	7.51	28.71	1.98	14.13	85.7	82.81	1.39	46.12	0.58
Shrub												
Choerophryne gunneri (22)	16.40	0.42	13.53	8.50	30.87	1.44	10.30	4.28	141.50	2.52	17.92	0.20
Choerophryne sp 1 (2)	19.32	0.60	17.75	9.18	19.92	1.44	10.28	7.19	156	2.72	26.67	0.17
Choerophryne sp 2 (7)	19.38	0.47	2.86	1.47	28.19	1.30	9.25	5.55	168.71	2.94	20.35	0.21
Choerophryne sp 3 (6)	15.85	0.37	19.21	12.09	32.20	1.68	11.98	4.29	173.73	3.01	22.30	0.25
Cophixalus cheesmanae (16)	26.25	2.09	30.41	12.21	31.85	1.93	13.76	33.4	124.17	2.13	35.53	0.41

Table 4S. (Continued) Performance and SVL means of species separated by ecomorph. Units are located under each heading.

Cophixalus sp. 1 (23)	16.26	0.60	17.12	10.58	31.91	1.71	12.21	7.03	140.24	2.42	19.92	0.24
Cophixalus linnaeus (1)	13.25	0.38	12.22	9.08	26.21	1.43	10.21	2.61	173.12	3.14	26.06	0.41
Cophixalus variabilis (45)	13.90	0.36	18.55	12.20	40.65	1.77	12.66	4.92	113.70	2.83	23.68	0.37
Cophixalus verrucosus (30)	19.96	1.07	33.54	17.01	39.69	2.06	14.72	23.0	113.42	2.87	25.06	0.45
Arboreal												
Oreophryne geislerorum (3)	23.40	2.20	30.50	13.02	28.21	1.87	13.34	26.5	131.33	2.29	32.50	0.35
Oreophryne loriae (18)	24.75	1.25	29.23	12.42	30.02	1.86	13.30	16.5	145.13	2.76	34.19	0.35
Oreophryne sp (9)	24.91	1.56	23.50	9.43	29.93	1.64	11.75	20.2	154.78	2.78	22.35	0.30

Table 5S. Parameter estimated for the microhabitat model for the morphological measurements. Strength of selection (α) and noise (σ) are shown for morphological variables for which the microhabitat model performed best. Estimated optimal values (Θ) for each ecomorph are shown for size-adjusted femur length (mm), size-adjusted tibiofibula length (mm), size-adjusted tarsus length (mm), size-adjusted radioulna (mm), and size-adjusted toe pad width (mm). Only σ is displayed for foot length, head length, head width, and hand length as these variables were not explained by a selection- based model. Bootstrap 95% confidence intervals for parameter estimates are in parentheses.

	Femur	Tibiofibula	Tarsus	Foot	Head Length	Head Width	Radioulna	Hand	Toe Pad
α	10.6 (6.91, 14.3)	12 (6, 18.1)	20.1 (15.3, 24.8)				15.4 (10.9, 19.8)		24.5 (20.1, 35)
σ	0.03 (0.02, 0.04)	0.12 (0.06, 0.18)	0.19 (0.15, 0.24)	0.019 (0.018, 0.02)	0.24 (0.23, 0.25)	0.01 (0.009, 0.01)	0.06 (0.04, 0.09)	0.02 (0.02, 0.02)	0.63 (0.45, 0.82)
$\Theta_{Subterranean}$	0.40 (0.39, 0.41)	0.35 (0.34, 0.36)	0.25 (0.24, 0.25)				0.17 (0.16, 0.17)		0.03 (0.03, 0.03)
Θ_{Ground}	0.49 (0.48 0.49)	0.51 (0.50, 0.51)	0.31 (0.30, 0.31)				0.23 (0.22, 0.23)		0.04 (0.04, 0.04)
$\Theta_{Semi-aquatic}$	0.48 (0.47, 0.49)	0.42 (0.41, 0.43)	0.26 (0.26, 0.27)				0.19 (0.19, 0.19)		0.05 (0.05, 0.05)
Θ_{Shrub}	0.46 (0.45, 0.47)	0.45 (0.44, 0.45)	0.28 (0.28, 0.29)				0.23 (0.23, 0.24)		0.05 (0.05, 0.05)
Θ_{Tree}	0.41 (0.40 , 0.42)	0.41 (0.40, 0.42)	0.28 (0.27, 0.28)				0.21 (0.21, 0.22)		0.06 (0.06, 0.06)

Table 6S. Analysis of Variance for relative jump performance as a function of ecomorph.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Ecomorph	4	3466	866.6	6.893	2.33e-05
Residuals	369	46390	125.7		

Table 7S. Circular Analysis of Variance with high concentration F-test for relative cling performance as a function of ecomorph.

	Df	SS	MS	F	P
Between	4	26.51	6.62	17.31	8.71e-13
Within	299	212.38	0.71	NA	NA
Total	303	238.89	0.78	NA	NA

Table 8S. Analysis of Variance for relative swim performance as a function of ecomorph.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Ecomorph	4	14.02	3.504	24.89	<2e-16
Residuals	250	35.20	0.141		

Table 9S. Analysis of Variance of relative jump performance as a function of tibiofibula morphology and ecomorph.

Response: Jump Performance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Tibiofibula	1	260.5	260.50	7.15	0.013
Residuals	24	874.0	36.41		

Table 10S. Analysis of Variance of relative jump performance as a function of toe-pad width morphology and ecomorph

Response: Jump Performance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Toe-pad	1	187.51	187.50	8.02	0.01
Ecomorph	4	479.64	119.91	5.13	0.005
Residuals	20	467.36	23.36		

Table 11S. Analysis of Variance of relative cling performance as a function of toe-pad morphology and ecomorph.

Response: Cling Performance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)

Toe-pad	1	10.20	10.20	49.82	5.76e-07
Ecomorph	4	3.63	0.90	4.43	0.0093
Residuals	21	4.30	0.20		

Table 12S. Analysis of Variance of relative cling performance as a function of foot morphology and ecomorph.
Response: Cling Performance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Foot	1	2.69	2.69	16.68	0.00069
Ecomorph	4	9.35	2.33	14.46	1.87e-05
Foot:Ecomorph	3	3.18	1.06	6.57	0.0034
Residuals	18	2.90	0.16		

Table 13S. Analysis of Variance of relative swim performance as a function of tibiofibula morphology and ecomorph.
Response: Swim Performance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Tibiofibula	1	0.13	0.13	36.47	6.64e-06
Ecomorph	4	0.10	0.025	6.69	0.0013
Residuals	20	0.075	0.0037		

Table 14S. Analysis of Variance of relative swim performance as a function of tarsus morphology and ecomorph.
Response: Swim Performance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Tarsus	1	0.10	0.10	18.98	0.00030
Ecomorph	4	0.10	0.02	4.62	0.008
Residuals	20	0.10	0.005		

Table 15S. Analysis of Variance of relative swim performance as a function of foot morphology and ecomorph.
Response: Swim Performance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Foot	1	0.16	0.16	45.25	1.51e-06
Ecomorph	4	0.07	0.017	4.72	0.0075
Residuals	20	0.07	0.0037		

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